IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Tanel TENSON et al.	Group Art Unit: 1636
Application No.: 10/531,870	Examiner: N. Vogel
Filed: April 19, 2005	Confirmation No.: 5979
For: SELECTION SYSTEM CONTAINING NON-ANTIBIOTIC RESISTANCE SELECTION MARKER	

DECLARATION PURSUANT TO 37 C.F.R. §§ 1.821-1.825

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Lisa E. Stahl, declare as follows:

That the content of the paper copy of the Sequence Listing filed concurrently herewith and the content of the computer readable copy of the Sequence Listing submitted herewith, in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same in compliance with § 1.821(f).

That the submission, filed in accordance with 37 C.F.R. § 1.821(g)[or (h)], herein does not include new matter [or go beyond the disclosure in the international application].

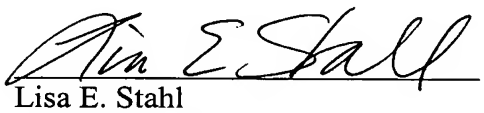
I hereby declare that all statements made herein of my own knowledge are true and that all statements were made on information and belief and are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of

Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date: September 4, 2007

By: 

Lisa E. Stahl
Registration No. 56,704

P.O. Box 1404
Alexandria, VA 22313-1404
703 838 6609

L-ribulose-5-phosphate 4-epimerase protein sequences from different bacteria.

Blast query is L-ribulose-5-phosphate 4-epimerase protein sequence encoded by E. coli *araD* cds.

1. Shigella flexneri

```
Length=231

 Score =  474 bits (1220),  Expect = 2e-132
 Identities = 230/231 (99%), Positives = 231/231 (100%), Gaps = 0/231 (0%)

Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            ML+DLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS
Sbjct  1    MLKDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
            IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD
Sbjct  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
            YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA
Sbjct  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231
            EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ
Sbjct  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231

LOCUS       NP_706015               231 aa            linear   BCT 03-APR-2006
DEFINITION  L-ribulose-5-phosphate 4-epimerase [Shigella flexneri 2a str. 301].
ACCESSION   NP_706015
VERSION     NP_706015.1  GI:24111505
DBSOURCE    REFSEQ: accession NC_004337.1
KEYWORDS    .
SOURCE      Shigella flexneri 2a str. 301
  ORGANISM  Shigella flexneri 2a str. 301
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Shigella.
REFERENCE   1  (residues 1 to 231)
```

AUTHORS Jin,Q., Yuan,Z., Xu,J., Wang,Y., Shen,Y., Lu,W., Wang,J., Liu,H., Yang,J., Yang,F., Zhang,X., Zhang,J., Yang,G., Wu,H., Qu,D., Dong,J., Sun,L., Xue,Y., Zhao,A., Gao,Y., Zhu,J., Kan,B., Ding,K., Chen,S., Cheng,H., Yao,Z., He,B., Chen,R., Ma,D., Qiang,B., Wen,Y., Hou,Y. and Yu,J.

TITLE Genome sequence of Shigella flexneri 2a: insights into pathogenicity through comparison with genomes of Escherichia coli K12 and O157

JOURNAL Nucleic Acids Res. 30 (20), 4432-4441 (2002)

PUBMED 12384590

REFERENCE 2 (residues 1 to 231)

CONSRTM NCBI Genome Project

TITLE Direct Submission

JOURNAL Submitted (18-OCT-2002) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

REFERENCE 3 (residues 1 to 231)

AUTHORS Jin,Q., Shen,Y., Wang,J.H., Liu,H., Yang,J., Yang,F., Zhang,X.B., Zhang,J.Y., Yang,G.W., Wu,H.T., Dong,J., Sun,L.L., Xue,Y., Zhao,A.L., Gao,Y.S., Zhu,J.P., Chen,S.X., Yao,Z.J., Wang,Y., Lu,W.C., Qiang,B.Q., Wen,Y.M. and Hou,Y.D.

TITLE Direct Submission

JOURNAL Submitted (21-MAY-2001) State Key Laboratory for Moleclular Virology and Genetic Engineering, Microbial Genome Center of Chinese Ministry of Public Health, 6 Rongjing Eastern Street, BDA, Beijing 100176, P.R.China

COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from AAN41722.
Method: conceptual translation.

LOCUS NP_835797 231 aa linear BCT 30-APR-2007

DEFINITION L-ribulose-5-phosphate 4-epimerase [Shigella flexneri 2a str. 2457T].

ACCESSION NP_835797

VERSION NP_835797.1 GI:30061626

DBSOURCE REFSEQ: accession NC 004741.1
KEYWORDS .
SOURCE Shigella flexneri 2a str. 2457T
ORGANISM Shigella flexneri 2a str. 2457T
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; Shigella.
REFERENCE 1 (residues 1 to 231)
AUTHORS Wei,J., Goldberg,M.B., Burland,V., Venkatesan,M.M., Deng,W.,
Fournier,G., Mayhew,G.F., Plunkett,G. III, Rose,D.J., Darling,A.,
Mau,B., Perna,N.T., Payne,S.M., Runyen-Janecky,L.J., Zhou,S.,
Schwartz,D.C. and Blattner,F.R.
TITLE Complete Genome Sequence and Comparative Genomics of Shigella
flexneri Serotype 2a Strain 2457T
JOURNAL Infect. Immun. 71 (5), 2775-2786 (2003)
PUBMED 12704152
REFERENCE 2 (residues 1 to 231)
CONSRTM NCBI Genome Project
TITLE Direct Submission
JOURNAL Submitted (23-APR-2003) National Center for Biotechnology
Information, NIH, Bethesda, MD 20894, USA
REFERENCE 3 (residues 1 to 231)
AUTHORS Wei,J., Goldberg,M.B., Burland,V., Venkatesan,M.M., Deng,W.,
Fournier,G., Mayhew,G.F., Plunkett,G. III, Rose,D.J., Darling,A.,
Mau,B., Perna,N.T., Payne,S.M., Runyen-Janecky,L.J., Zhou,S.,
Schwartz,D.C. and Blattner,F.R.
TITLE Direct Submission
JOURNAL Submitted (13-JUN-2002) Genetics Laboratory, University of
Wisconsin - Madison, 445 Henry Mall, Madison, WI 53706, USA
COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of
the sequence, but has not yet been subject to final review. The
reference sequence was derived from AAP15602.
Method: conceptual translation.

2. Salmonella typhimurium

```
Length=248

 Score =  404 bits (1038),  Expect = 2e-111
 Identities = 196/208 (94%), Positives = 200/208 (96%), Gaps = 0/208 (0%)

Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGV VIKPSGVDYSVMTADDMVVVS
Sbjct  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVLVIKPSGVDYSVMTADDMVVVS  60

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
            +E+GEVVEG KKPSSDTPTHRLLYQAFP+IGGIVHTHSRHATIWAQAGQ IPATGTTHAD
Sbjct  61   LESGEVVEGHKKPSSDTPTHRLLYQAFPTIGGIVHTHSRHATIWAQAGQPIPATGTTHAD  120

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
            YFYGTIPCTRKMT+AEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA
Sbjct  121  YFYGTIPCTRKMTEAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLP  208
            EDAVHNAIVLEEVAYMGIFCR L    P
Sbjct  181  EDAVHNAIVLEEVAYMGIFCRHLRRSCP  208


LOCUS       AAA27025                 248 aa            linear   BCT 11-MAR-1994
DEFINITION  L-ribulose-5-phosphate 4-epimerase.
ACCESSION   AAA27025
VERSION     AAA27025.1  GI:153869
DBSOURCE    locus STYARABAD accession M11047.1
KEYWORDS    .
SOURCE      Salmonella typhimurium
  ORGANISM  Salmonella typhimurium
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
```

Enterobacteriaceae; Salmonella.

REFERENCE 3 (residues 1 to 4790)
AUTHORS Lin,H.C., Lei,S.P., Studnicka,G. and Wilcox,G.
TITLE The araBAD operon of Salmonella typhimurium LT2. III. Nucleotide sequence of araD and its flanking regions, and primary structure of its product, L-ribulose-5-phosphate 4-epimerase
JOURNAL Gene 34 (1), 129-134 (1985)
PUBMED 3891514
COMMENT The sequence preceding araB coding region is part of the controlling region between the araC gene and araBAD operon. A potential ribosome binding site for the araB gene is located at positions 109-112. A 10-bp intercistronic region is located between the araB and araA genes. A potential ribosome binding site, 'taagga', is located 7 bp distal from the start codon of araA. The site overlaps the stop codon of araB .
A 143-bp intercistronic region exists between the araA and araD genes. The presumed ribosome binding site for araD is located at positions 3473-3475. This region contains several short complementary repeated sequences which can form stable stem-loop secondary structures. There is also a stem-loop structure 80 bp beyond the stop codon of araD which is followed by an A+T-rich sequence.
Method: conceptual translation.

Length=231

Score = 462 bits (1189), Expect = 7e-129
Identities = 223/231 (96%), Positives = 228/231 (98%), Gaps = 0/231 (0%)

```
Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGV VIKPSGVDYSVMTADDMVVVS
Sbjct  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVLVIKPSGVDYSVMTADDMVVVS  60

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
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```
            +E+GEVVEG KKPSSDTPTHRLLYQAFP+IGGIVHTHSRHATIWAQAGQ IPATGTTHAD
Sbjct  61   LESGEVVEGHKKPSSDTPTHRLLYQAFPTIGGIVHTHSRHATIWAQAGQPIPATGTTHAD  120

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
            YFYGTIPCTRKMT+AEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA
Sbjct  121  YFYGTIPCTRKMTEAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231
            EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQ+LLDKHYLRKHGAKAYYGQ
Sbjct  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQSLLDKHYLRKHGAKAYYGQ  231
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LOCUS       NP_459106                231 aa            linear   BCT 13-APR-2007
DEFINITION  L-ribulose-5-phosphate 4-epimerase [Salmonella typhimurium LT2].
ACCESSION   NP_459106
VERSION     NP_459106.1  GI:16763491
DBSOURCE    REFSEQ: accession NC_003197.1
KEYWORDS    .
SOURCE      Salmonella typhimurium LT2
  ORGANISM  Salmonella typhimurium LT2
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Salmonella.
REFERENCE   1  (residues 1 to 231)
  AUTHORS   McClelland,M., Sanderson,K.E., Spieth,J., Clifton,S.W.,
            Latreille,P., Courtney,L., Porwollik,S., Ali,J., Dante,M., Du,F.,
            Hou,S., Layman,D., Leonard,S., Nguyen,C., Scott,K., Holmes,A.,
            Grewal,N., Mulvaney,E., Ryan,E., Sun,H., Florea,L., Miller,W.,
            Stoneking,T., Nhan,M., Waterston,R. and Wilson,R.K.
  TITLE     Complete genome sequence of Salmonella enterica serovar Typhimurium
            LT2
  JOURNAL   Nature 413 (6858), 852-856 (2001)
   PUBMED   11677609
REFERENCE   2  (residues 1 to 231)
  CONSRTM   NCBI Genome Project
  TITLE     Direct Submission
```

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  JOURNAL   Submitted (10-SEP-2004) National Center for Biotechnology
            Information, NIH, Bethesda, MD 20894, USA
REFERENCE   3  (residues 1 to 231)
  CONSRTM   NCBI Microbial Genomes Annotation Project
  TITLE     Direct Submission
  JOURNAL   Submitted (06-NOV-2001) National Center for Biotechnology
            Information, NIH, Bethesda, MD 20894, USA
COMMENT     VALIDATED REFSEQ: This record has undergone preliminary review of
            the sequence, but has not yet been subject to final review. The
            reference sequence was derived from AAL19065.
            Method: conceptual translation.
--------------------------------------------------------------------------------
LOCUS       AAL19065                 231 aa            linear   BCT 09-AUG-2005
DEFINITION  L-ribulose-5-phosphate 4-epimerase [Salmonella typhimurium LT2].
ACCESSION   AAL19065
VERSION     AAL19065.1  GI:16418599
DBSOURCE    accession AE008698.1
KEYWORDS    .
SOURCE      Salmonella typhimurium LT2
  ORGANISM  Salmonella typhimurium LT2
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Salmonella.
REFERENCE   1  (residues 1 to 231)
  AUTHORS   McClelland,M., Sanderson,K.E., Spieth,J., Clifton,S.W.,
            Latreille,P., Courtney,L., Porwollik,S., Ali,J., Dante,M., Du,F.,
            Hou,S., Layman,D., Leonard,S., Nguyen,C., Scott,K., Holmes,A.,
            Grewal,N., Mulvaney,E., Ryan,E., Sun,H., Florea,L., Miller,W.,
            Stoneking,T., Nhan,M., Waterston,R. and Wilson,R.K.
  TITLE     Complete genome sequence of Salmonella enterica serovar Typhimurium
            LT2
  JOURNAL   Nature 413 (6858), 852-856 (2001)
   PUBMED   11677609
REFERENCE   2  (residues 1 to 231)
  CONSRTM   The Salmonella typhimurium Genome Sequencing Project
  TITLE     Direct Submission
  JOURNAL   Submitted (29-MAR-2001) Genome Sequencing Center, Department of
```

Genetics, Washington University School of Medicine, 4444 Forest Park Boulevard, St. Louis, MO 63108, USA

COMMENT Supported by NIH grant 5U 01 AI43283

Coding sequences below are predicted from manually evaluated computer analysis, using similarity information and the programs; GLIMMER; http://www.tigr.org/softlab/glimmer/glimmer.html and GeneMark; http://opal.biology.gatech.edu/GeneMark/

EC numbers were kindly provided by Junko Yabuzaki and the Kyoto Encyclopedia of Genes and Genomes; http://www.genome.ad.jp/kegg/, and Pedro Romero and Peter Karp at EcoCyc; http://ecocyc.PangeaSystems.com/ecocyc/

The analyses of ribosome binding sites and promoter binding sites were kindly provided by Heladia Salgado, Julio Collado-Vides and ReguonDB; http://kinich.cifn.unam.mx:8850/db/regulondb_intro.frameset

This sequence was finished as follows unless otherwise noted: all regions were double stranded, sequenced with an alternate chemistries or covered by high quality data (i.e., phred quality >= 30); an attempt was made to resolve all sequencing problems, such as compressions and repeats; all regions were covered by sequence from more than one m13 subclone.

3. Salmonella enterica

```
Length=231

 Score =  456 bits (1172),  Expect = 6e-127
 Identities = 220/231 (95%), Positives = 225/231 (97%), Gaps = 0/231 (0%)

Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            MLEDLK QVLEANLALPKHNLVTLTWGNVSAVDRERGV VIKPSGVDYSVMTADDMVVVS
Sbjct  1    MLEDLKHQVLEANLALPKHNLVTLTWGNVSAVDRERGVLVIKPSGVDYSVMTADDMVVVS  60

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
            +ETGEVVEG KKPSSDTPTHRLLYQAFP+IG IVHTHSRHATIWAQAGQ IPATGTTHAD
Sbjct  61   LETGEVVEGHKKPSSDTPTHRLLYQAFPTIGSIVHTHSRHATIWAQAGQPIPATGTTHAD  120

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
            YFYGTIPCTRKMT+AEINGEYEWETGNVIVE FEKQGI+AAQMPGVLVHSHGPFAWGKNA
Sbjct  121  YFYGTIPCTRKMTEAEINGEYEWETGNVIVEAFEKQGINAAQMPGVLVHSHGPFAWGKNA  180

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231
            EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQ+LLDKHYLRKHGAKAYYGQ
Sbjct  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQSLLDKHYLRKHGAKAYYGQ  231

LOCUS       NP_454713                231 aa            linear   BCT 04-APR-2006
DEFINITION  L-ribulose-5-phosphate 4-epimerase [Salmonella enterica subsp.
            enterica serovar Typhi str. CT18].
ACCESSION   NP_454713
VERSION     NP_454713.1  GI:16759096
DBSOURCE    REFSEQ: accession NC_003198.1
KEYWORDS    .
SOURCE      Salmonella enterica subsp. enterica serovar Typhi str. CT18
  ORGANISM  Salmonella enterica subsp. enterica serovar Typhi str. CT18
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Salmonella; Salmonella enterica subsp. enterica
```

serovar Typhi.
REFERENCE 1 (residues 1 to 231)
AUTHORS Parkhill,J., Dougan,G., James,K.D., Thomson,N.R., Pickard,D., Wain,J., Churcher,C., Mungall,K.L., Bentley,S.D., Holden,M.T.G., Sebaihia,M., Baker,S., Basham,D., Brooks,K., Chillingworth,T., Connerton,P., Cronin,A., Davis,P., Davies,R.M., Dowd,L., White,N., Farrar,J., Feltwell,T., Hamlin,N., Haque,A., Hien,T.T., Holroyd,S., Jagels,K., Krogh,A., Larsen,T.S., Leather,S., Moule,S., O'Gaora,P., Parry,C., Quail,M., Rutherford,K., Simmonds,M., Skelton,J., Stevens,K., Whitehead,S. and Barrell,B.G.
TITLE Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18
JOURNAL Nature 413 (6858), 848-852 (2001)
PUBMED 11677608
REFERENCE 2 (residues 1 to 231)
AUTHORS Parkhill,J.
TITLE Direct Submission
JOURNAL Submitted (25-OCT-2001) Submitted on behalf of the Salmonella sequencing team, Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK
REFERENCE 3 (residues 1 to 231)
CONSRTM NCBI Genome Project
TITLE Direct Submission
JOURNAL Submitted (07-OCT-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from CAD01258.

4. Yersinia pestis

```
Length=267

 Score =  365 bits (937),  Expect = 1e-99
 Identities = 173/231 (74%), Positives = 197/231 (85%), Gaps = 0/231 (0%)

Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            ML +LK+QVL ANLALP+HNLVT TWGNVSA+DR++G+ VIKPSGV+Y+ MT DDMVVV
Sbjct  37   MLNELKQQVLAANLALPRHNLVTFTWGNVSAIDRQKGLLVIKPSGVEYASMTLDDMVVVE  96

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
            +E+G VVEG+KKPSSDT THR+LY  FP IGGIVHTHSRHATIWAQAG  +PA GTTHAD
Sbjct  97   LESGNVVEGSKKPSSDTDTHRVLYLNFPQIGGIVHTHSRHATIWAQAGLDLPAWGTTHAD  156

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
            YFYG+IPCTR MT  EI G YEWETGNVIV+TF ++GI    +P VLV+SHGPFAWG +A
Sbjct  157  YFYGSIPCTRLMTHEEIAGRYEWETGNVIVDTFHERGITPDAVPAVLVNSHGPFAWGSSA  216

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231
            E+AVHNA+VLEE+AYMGIF RQL PQL DMQ  LLDKHYLRKHG  AYYGQ
Sbjct  217  ENAVHNAVVLEELAYMGIFSRQLNPQLGDMQPQLLDKHYLRKHGKDAYYGQ  267


LOCUS       NP_669385                267 aa            linear   BCT 26-JAN-2006
DEFINITION  L-ribulose-5-phosphate 4-epimerase [Yersinia pestis KIM].
ACCESSION   NP_669385
VERSION     NP_669385.1  GI:22125962
DBSOURCE    REFSEQ: accession NC_004088.1
KEYWORDS    .
SOURCE      Yersinia pestis KIM
  ORGANISM  Yersinia pestis KIM
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Yersinia.
```

REFERENCE 1 (residues 1 to 267)
AUTHORS Deng,W., Burland,V., Plunkett,G. III, Boutin,A., Mayhew,G.F., Liss,P., Perna,N.T., Rose,D.J., Mau,B., Zhou,S., Schwartz,D.C., Fetherston,J.D., Lindler,L.E., Brubaker,R.R., Plana,G.V., Straley,S.C., McDonough,K.A., Nilles,M.L., Matson,J.S., Blattner,F.R. and Perry,R.D.
TITLE Genome Sequence of Yersinia pestis KIM
JOURNAL J. Bacteriol. 184 (16), 4601-4611 (2002)
PUBMED 12142430
REFERENCE 2 (residues 1 to 267)
CONSRTM NCBI Genome Project
TITLE Direct Submission
JOURNAL Submitted (10-SEP-2004) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
REFERENCE 3 (residues 1 to 267)
AUTHORS Deng,W., Burland,V., Plunkett,G. III, Boutin,A., Mayhew,G.F., Liss,P., Perna,N.T., Rose,D.J., Mau,B., Zhou,S., Schwartz,D.C., Fetherston,J.D., Lindler,L.E., Brubaker,R.R., Plana,G.V., Straley,S.C., McDonough,K.A., Nilles,M.L., Matson,J.S., Blattner,F.R. and Perry,R.D.
TITLE Direct Submission
JOURNAL Submitted (21-FEB-2002) Genetics, University of Wisconsin, 445 Henry Mall, Madison, WI 53706, USA
COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from AAM85636.
Method: conceptual translation.

5. Pasteurella multocida

```
Length=243

 Score =  352 bits (904),  Expect = 7e-96
 Identities = 166/231 (71%), Positives = 190/231 (82%), Gaps = 0/231 (0%)

Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            MLE+LK++V EANLALPK+ LVT TWGNVS +DRE+ + VIKPSGV+Y  MT +DMVVV
Sbjct  13   MLEELKQKVFEANLALPKYKLVTFTWGNVSGIDREKNLVVIKPSGVEYDTMTVEDMVVVD  72

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
            + TG+VVEG KKPSSDT TH  LY+ FPS+GGIVHTHSRHATIWAQAG+ + A GTTHAD
Sbjct  73   LFTGQVVEGNKKPSSDTATHLELYRQFPSLGGIVHTHSRHATIWAQAGEDLIAAGTTHAD  132

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
            YFYG+IPCTRKMT AEI GEYE ETG VIVETF  +GID   +P VLVHSHGPFAWG +
Sbjct  133  YFYGSIPCTRKMTPAEIQGEYELETGKVIVETFRVRGIDPKDVPAVLVHSHGPFAWGTDP  192

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231
            ++AVHNA+VLEE+ YM +F RQL P L  MQQ LLDKHYLRKHG  AYYGQ
Sbjct  193  DNAVHNAVVLEEIGYMNLFSRQLRPNLASMQQELLDKHYLRKHGKNAYYGQ  243


LOCUS       NP_246181               243 aa            linear   BCT 03-DEC-2005
DEFINITION  L-ribulose-5-phosphate 4-epimerase [Pasteurella multocida subsp.
            multocida str. Pm70].
ACCESSION   NP_246181
VERSION     NP_246181.1  GI:15603109
DBSOURCE    REFSEQ: accession NC_002663.1
KEYWORDS    .
SOURCE      Pasteurella multocida subsp. multocida str. Pm70
  ORGANISM  Pasteurella multocida subsp. multocida str. Pm70
            Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales;
```

Pasteurellaceae; Pasteurella.

REFERENCE 1 (residues 1 to 243)
AUTHORS May,B.J., Zhang,Q., Li,L.L., Paustian,M.L., Whittam,T.S. and Kapur,V.
TITLE Complete genomic sequence of Pasteurella multocida,Pm70
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 98 (6), 3460-3465 (2001)
PUBMED 11248100

REFERENCE 2 (residues 1 to 243)
CONSRTM NCBI Genome Project
TITLE Direct Submission
JOURNAL Submitted (13-SEP-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

REFERENCE 3 (residues 1 to 243)
CONSRTM NCBI Microbial Genomes Annotation Project
TITLE Direct Submission
JOURNAL Submitted (25-JUN-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from AAK03328.
Method: conceptual translation supplied by author.

6. Haemophilus influenzae

```
 Score =  348 bits (893),  Expect = 1e-94
 Identities = 167/231 (72%), Positives = 187/231 (80%), Gaps = 0/231 (0%)

Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            ML  LK++V EANLALPKH+LVT TWGNVSA+DRE+ + VIKPSGVDY VMT +DMVVV
Sbjct  13   MLAQLKKEVFEANLALPKHHLVTFTWGNVSAIDREKNLVVIKPSGVDYDVMTENDMVVVD  72

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
            + TG +VEG KKPSSDTPTH  LY+ FP IGGIVHTHSRHATIWAQAG  I   GTTH D
Sbjct  73   LFTGNIVEGNKKPSSDTPTHLELYRQFPHIGGIVHTHSRHATIWAQAGLDIIEVGTTHGD  132

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
            YFYGTIPCTR+MT  EI G YE ETG VIVETF  +GI+   +P VLVHSHGPFAWGK+A
Sbjct  133  YFYGTIPCTRQMTTKEIKGNYELETGKVIVETFLSRGIEPDNIPAVLVHSHGPFAWGKDA  192

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231
             +AVHNA+VLEEVAYM +F +QL P L  MQ+ LLDKHYLRKHG  AYYGQ
Sbjct  193  NNAVHNAVVLEEVAYMNLFSQQLNPYLSPMQKDLLDKHYLRKHGQNAYYGQ  243


LOCUS       H64108                  243 aa            linear   BCT 15-MAY-1998
DEFINITION  L-ribulose-phosphate 4-epimerase (EC 5.1.3.4) - Haemophilus
            influenzae (strain Rd KW20).
ACCESSION   H64108
VERSION     H64108  GI:1074955
DBSOURCE    pir: locus H64108;

            summary: #length 243 #molecular-weight 27241 #checksum 6284
            ;
            genetic: #start_codon GTG
```

```
            ;
            superfamily: L-ribulose-phosphate 4-epimerase
            ;
            PIR dates: 18-Aug-1995 #sequence_revision 18-Aug-1995 #text_change
            15-May-1998
            .
KEYWORDS    arabinose metabolism; isomerase; zinc.
SOURCE      Haemophilus influenzae
  ORGANISM  Haemophilus influenzae
            Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales;
            Pasteurellaceae; Haemophilus.
REFERENCE   1  (residues 1 to 243)
  AUTHORS   Fleischmann,R.D., Adams,M.D., White,O., Clayton,R.A.,
            Kirkness,E.F., Kerlavage,A.R., Bult,C.J., Tomb,J.F.,
            Dougherty,B.A., Merrick,J.M., McKenney,K., Sutton,G., FitzHugh,W.,
            Fields,C., Gocayne,J.D., Scott,J., Shirley,R., Liu,L.I., Glodek,A.,
            Kelley,J.M., Weidman,J.F., Phillips,C.A., Spriggs,T., Hedblom,E.,
            Cotton,M.D., Utterback,T.R., Hanna,M.C., Nguyen,D.T., Saudek,D.M.,
            Brandon,R.C., Fine,L.D., Fritchman,J.L., Fuhrmann,J.L.,
            Geoghagen,N.S.M., Gnehm,C.L., McDonald,L.A., Small,K.V.,
            Fraser,C.M., Smith,H.O. and Venter,J.C.
  TITLE     Whole-genome random sequencing and assembly of Haemophilus
            influenzae Rd
  JOURNAL   Science 269 (5223), 496-512 (1995)
```

7. Oceanobacillus iheyensis

```
Length=231

 Score =  298 bits (763),  Expect = 2e-79
 Identities = 142/231 (61%), Positives = 170/231 (73%), Gaps = 1/231 (0%)

Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            MLE LK +V EANL LPK  LV  TWGN SA DRE G+FVIKPSGVDY  M A DMVVV
Sbjct  1    MLEQLKEEVFEANLDLPKQGLVKYTWGNASAFDRESGLFVIKPSGVDYKTMKASDMVVVD  60

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
            ++ G VVEG   PSSDT TH +LY+ +P +GGIVHTHS  AT+WAQAG  +P  GTTHAD
Sbjct  61   LD-GNVVEGELNPSSDTATHAVLYKRYPELGGIVHTHSTWATVWAQAGLDVPVMGTTHAD  119

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
             FYG +PCTR +T  EI+  YE ETG VI+ETFE++G+D   +PGVL+H H PF WGK+
Sbjct  120  TFYGAVPCTRFLTQEEIDRGYEAETGRVIIETFEERGLDVFAIPGVLLHGHAPFTWGKDV  179

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231
            + AV N++VLEEVA M +F R+L    P++   +LDKHYLRKHG  AYYGQ
Sbjct  180  QSAVVNSVVLEEVAKMNLFARELNRFAPELPDRILDKHYLRKHGGDAYYGQ  230

LOCUS       NP_693720               231 aa            linear   BCT 02-DEC-2005
DEFINITION  L-ribulose-5-phosphate 4-epimerase [Oceanobacillus iheyensis
            HTE831].
ACCESSION   NP_693720
VERSION     NP_693720.1  GI:23100253
DBSOURCE    REFSEQ: accession NC_004193.1
KEYWORDS    .
SOURCE      Oceanobacillus iheyensis HTE831
  ORGANISM  Oceanobacillus iheyensis HTE831
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Oceanobacillus.
REFERENCE   1  (residues 1 to 231)
```

AUTHORS Takami,H., Takaki,Y. and Uchiyama,I.
TITLE Genome sequence of Oceanobacillus iheyensis isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments
JOURNAL Nucleic Acids Res. 30 (18), 3927-3935 (2002)
PUBMED 12235376
REFERENCE 2
AUTHORS Lu,J., Nogi,Y. and Takami,H.
TITLE Oceanobacillus iheyensis gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge
JOURNAL FEMS Microbiol. Lett. 205 (2), 291-297 (2001)
PUBMED 11750818
REFERENCE 3 (residues 1 to 231)
CONSRTM NCBI Genome Project
TITLE Direct Submission
JOURNAL Submitted (10-SEP-2004) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
REFERENCE 4 (residues 1 to 231)
AUTHORS NCBI Microbial Genomes Annotation Project.
TITLE Direct Submission
JOURNAL Submitted (24-FEB-2003) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from BAC14754.
Method: conceptual translation.

8. Bacillus halodurans

```
Length=231

 Score =  293 bits (750),  Expect = 5e-78
 Identities = 146/231 (63%), Positives = 172/231 (74%), Gaps = 3/231 (1%)

Query  1    MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS  60
            MLE LK  V +ANL LPK+ LVT TWGNVS +DRE+G+ VIKPSGV+Y  M + DMVVV
Sbjct  1    MLEQLKETVFKANLYLPKYQLVTFTWGNVSGIDREKGLVVIKPSGVEYFEMKSKDMVVVD  60

Query  61   IETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD  120
            +E G +VEG  KPSSDTPTH  LY+AF  +GGIVHTHS  AT WAQAG+ IPA GTTHAD
Sbjct  61   LE-GNIVEGDLKPSSDTPTHLALYRAFDKVGGIVHTHSVWATAWAQAGKEIPAYGTTHAD  119

Query  121  YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFAWGKNA  180
            YF+GTIPCTR MT+ EI G+YE ETGNVIVETF  +  D   +PGVLVHSH PF WGK+
Sbjct  120  YFHGTIPCTRPMTETEILGDYEKETGNVIVETFRNK--DPMSIPGVLVHSHAPFVWGKDP  177

Query  181  EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ  231
             +AVH+A+VLEEVA M      ++ +   M   LLD+H+ RKHG  AYYGQ
Sbjct  178  MEAVHHAVVLEEVAKMAQKTLSISERTLPMDSVLLDRHFYRKHGQAAYYGQ  228


LOCUS       NP_242737               231 aa            linear   BCT 03-DEC-2005
DEFINITION  L-ribulose-5-phosphate 4-epimerase [Bacillus halodurans C-125].
ACCESSION   NP_242737
VERSION     NP_242737.1  GI:15614434
DBSOURCE    REFSEQ: accession NC_002570.2
KEYWORDS    .
SOURCE      Bacillus halodurans C-125
  ORGANISM  Bacillus halodurans C-125
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
```

REFERENCE 1 (residues 1 to 231)
AUTHORS Takami,H., Nakasone,K., Takaki,Y., Maeno,G., Sasaki,R., Masui,N., Fuji,F., Hirama,C., Nakamura,Y., Ogasawara,N., Kuhara,S. and Horikoshi,K.
TITLE Complete genome sequence of the alkaliphilic bacterium Bacillus halodurans and genomic sequence comparison with Bacillus subtilis
JOURNAL Nucleic Acids Res. 28 (21), 4317-4331 (2000)
PUBMED 11058132
REFERENCE 2 (residues 1 to 231)
AUTHORS Nakasone,K., Masui,N., Takaki,Y., Sasaki,R., Maeno,G., Sakiyama,T., Hirama,C., Fuji,F. and Takami,H.
TITLE Characterization and comparative study of the rrn operons of alkaliphilic Bacillus halodurans C-125
JOURNAL Extremophiles 4 (4), 209-214 (2000)
PUBMED 10972189
REFERENCE 3 (sites)
AUTHORS Takami,H. and Horikoshi,K.
TITLE Analysis of the genome of an alkaliphilic Bacillus strain from an industrial point of view
JOURNAL Extremophiles 4 (2), 99-108 (2000)
PUBMED 10805564
REFERENCE 4 (sites)
AUTHORS Takami,H., Takaki,Y., Nakasone,K., Sakiyama,T., Maeno,G., Sasaki,R., Hirama,C., Fuji,F. and Masui,N.
TITLE Genetic analysis of the chromosome of alkaliphilic Bacillus halodurans C-125
JOURNAL Extremophiles 3 (3), 227-233 (1999)
PUBMED 10484179
REFERENCE 5 (sites)
AUTHORS Takami,H., Masui,N., Nakasone,K. and Horikoshi,K.
TITLE Replication origin region of the chromosome of alkaliphilic Bacillus halodurans C-125
JOURNAL Biosci. Biotechnol. Biochem. 63 (6), 1134-1137 (1999)
PUBMED 10427704
REFERENCE 6 (sites)
AUTHORS Takami,H., Takaki,Y., Nakasone,K., Hirama,C., Inoue,A. and

Horikoshi,K.
TITLE Sequence analysis of a 32-kb region including the major ribosomal protein gene clusters from alkaliphilic Bacillus sp. strain C-125
JOURNAL Biosci. Biotechnol. Biochem. 63 (2), 452-455 (1999)
PUBMED 10192928
REFERENCE 7 (sites)
AUTHORS Takami,H., Nakasone,K., Ogasawara,N., Hirama,C., Nakamura,Y., Masui,N., Fuji,F., Takaki,Y., Inoue,A. and Horikoshi,K.
TITLE Sequencing of three lambda clones from the genome of alkaliphilic Bacillus sp. strain C-125
JOURNAL Extremophiles 3 (1), 29-34 (1999)
PUBMED 10086842
REFERENCE 8 (sites)
AUTHORS Takami,H., Nakasone,K., Hirama,C., Takaki,Y., Masui,N., Fuji,F., Nakamura,Y. and Inoue,A.
TITLE An improved physical and genetic map of the genome of alkaliphilic Bacillus sp. C-125
JOURNAL Extremophiles 3 (1), 21-28 (1999)
PUBMED 10086841
REFERENCE 9 (sites)
AUTHORS Takami,H.
TITLE Genome analysis of facultatively alkalihilic Bacillus halodurans C-125
JOURNAL (in) Extremophiles in deep-sea environments (Ed.);
. HORIKOSHI, K. TSUJII;
: 249-284; Springer-Verlag (1999)
REFERENCE 10 (sites)
AUTHORS Takami,H. and Horikoshi,K.
TITLE Reidentification of facultatively alkaliphilic Bacillus sp. C-125 to Bacillus halodurans
JOURNAL Biosci. Biotechnol. Biochem. 63, 943-945 (1999)
REFERENCE 11 (residues 1 to 231)
CONSRTM NCBI Genome Project
TITLE Direct Submission
JOURNAL Submitted (13-SEP-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

REFERENCE 12 (residues 1 to 231)
CONSRTM NCBI Microbial Genomes Annotation Project
TITLE Direct Submission
JOURNAL Submitted (25-JUN-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
REFERENCE 13 (residues 1 to 231)
AUTHORS Takami,H. and Takaki,Y.
TITLE Direct Submission
JOURNAL Submitted (22-MAR-2000) Japan Marine Science and Technology Center, Deep-sea Microorganisms Research Group, 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan
COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from BAB05590.

Microbiology (1997), 143, 957–969 Printed in Great Britain

The *Bacillus subtilis* L-arabinose (*ara*) operon: nucleotide sequence, genetic organization and expression

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[2] The Rockefeller University, Laboratory of Microbiology, 1230 York Avenue, New York, NY 10021-6399, USA

The *Bacillus subtilis* L-arabinose metabolic genes *araA*, *araB* and *araD*, encoding L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively, have been cloned previously and the products of *araB* and *araD* were shown to be functionally homologous to their *Escherichia coli* counterparts by complementation experiments. Here we report that *araA*, *araB* and *araD*, whose inactivation leads to an Ara phenotype, are the first three ORFs of a nine cistron transcriptional unit with a total length of 11 kb. This operon, called *ara*, is located at about 256° on the *B. subtilis* genetic map and contains six new genes named *araL*, *araM*, *araN*, *araP*, *araQ* and *abfA*. Expression of the *ara* operon is directed by a strong σ^A-like promoter identified within a 150 bp DNA fragment upstream from the translation start site of *araA*. Analysis of the sequence of the *ara* operon showed that the putative products of *araN*, *araP* and *araQ* are homologous to bacterial components of binding-protein-dependent transport systems and *abfA* most probably encodes an α-L-arabinofuranosidase. The functions of *araL* and *araM* are unknown. An *in vitro*-constructed insertion-deletion mutation in the region downstream from *araD* allowed us to demonstrate that *araL*, *araM*, *araN*, *araP*, *araQ* and *abfA* are not essential for L-arabinose utilization. Studies with strains bearing transcriptional fusions of the operon to the *E. coli lacZ* gene revealed that expression from the *ara* promoter is induced by L-arabinose and repressed by glucose.

Keywords: *Bacillus subtilis*, L-arabinose (*ara*) operon, expression, catabolite repression

INTRODUCTION

Bacillus subtilis, an endospore-forming Gram-positive bacterium, is able to grow on L-arabinose as sole carbon source. L-Arabinose residues are found widely distributed among many heteropolysaccharides of different plant tissues, such as arabinans, arabinogalactans, xylans and arabinoxylans. *Bacillus* species in their natural reservoir, the soil, participate in the early stages of plant material decomposition and *B. subtilis* secretes three enzymes, an endo-arabanase and two arabinosidases, capable of releasing arabinosyl oligomers and L-arabinose from plant cell walls (Kaji & Saheki, 1975; Weinstein & Albersheim, 1979). The pathway of L-arabinose utilization in *B. subtilis* has been described by Lepesant & Dedonder (1967a). After entering the cell, L-arabinose is sequentially converted to L-ribulose, L-ribulose 5-phosphate, and D-xylulose 5-phosphate by the action of L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively. D-Xylulose 5-phosphate is further catabolized through the pentose phosphate pathway. Mutants unable to use L-arabinose as sole carbon source, deficient in one of the three enzymes involved in L-arabinose catabolism, have been characterized, as well as constitutive mutants for

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‡**Present address:** Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA.

Abbreviations: Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Sp, spectinomycin.

The accession numbers for the nucleotide sequences reported in this paper are X89408 (*araA, B* and *D*) and X89810 (*araL, M, N, P, Q* and *abfA*).

all three enzymes (Lepesant & Dedonder, 1967a, b). The synthesis of these enzymes was shown to be inducible by L-arabinose and the isomerase activity is subjected to catabolite repression by glucose and glycerol (Lepesant & Dedonder, 1967a).

A collection of Ara$^-$ *B. subtilis* mutants was isolated, biochemically characterized and the three metabolic genes, *araA*, *araB* and *araD*, encoding L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively, were identified and mapped between *aroG* and *leuA*, at about 256° on the *B. subtilis* genetic map (Paveia & Archer, 1992a, b). Two additional classes of mutations affecting L-arabinose utilization were identified; one included mutations conferring an Ara$^-$ phenotype to strains bearing the *araA*, *araB* and *araD* wild-type alleles (Paveia & Archer, 1992a, b), and another comprised mutants showing constitutive expression of the three genes (Sá-Nogueira *et al.*, 1988). These mutations were mapped between the *cysB* and *hisA* markers, at about 294° on the *B. subtilis* genetic map, and define another *ara* locus named *araC*. Expression of L-arabinose isomerase is severely repressed during growth in media containing L-arabinose plus glucose. Since L-arabinose isomerase expression is still regulated by catabolite repression in strains which contain constitutive mutations (*araC*^{c}), L-arabinose transport does not play a major role in catabolite repression of expression of the metabolic enzymes (Sá-Nogueira *et al.*, 1988). The products of the previously cloned genes *araA*, *araB* and *araD* were shown in complementation experiments to be functionally homologous to their *Escherichia coli* counterparts. Transformation experiments involving defined restriction fragments from the cloned genes showed that they are adjacent and probably constitute an operon with the order *araABD* (Sá-Nogueira & Lencastre, 1989), unlike the *araBAD* order found in the *E. coli* operon (Englesberg *et al.*, 1969).

In this communication we report the cloning of an additional 7·1 kb chromosomal fragment, located downstream from *araD* and the nucleotide sequence of over 11 kb. This region contains a cluster of nine genes: the metabolic genes *araA*, *araB* and *araD*, and six new genes named *araL*, *araM*, *araN*, *araP*, *araQ* and *abfA*. We have demonstrated that all genes comprise a single transcriptional unit, called the *ara* operon, whose expression is directed by a single σ^A-type promoter identified within a 150 bp DNA fragment upstream from the translation start site of *araA*. The *araN*, *araP* and *araQ* gene products are likely components of a binding-protein-dependent transport system and *abfA* most probably encodes an α-L-arabinofuranosidase. In this study we define the promoter region of the *ara* operon and examine its expression and regulation using transcriptional fusions of this operon to the *E. coli lacZ* gene. These results indicate that the *ara* operon is regulated at the transcriptional level because expression from the *ara* promoter is induced by L-arabinose and repressed by glucose.

METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed on Table 1. *E. coli* DH5α (Gibco/BRL) was used as a host for all plasmids and *E. coli* DH5α F′ (BRL) for the propagation and amplification of recombinant M13 bacteriophages. *E. coli* strains were grown on LB (Luria–Bertani medium; Miller, 1972). Ampicillin (Ap, 75 μg ml^{-1}), chloramphenicol (Cm, 15 μg ml^{-1}), X-gal (40 μg ml^{-1}) or IPTG (1 mM) were added as appropriate. *B. subtilis* strains were grown on LB, SP medium (Martin *et al.*, 1987) or minimal C medium (Pascal *et al.*, 1971). Cm (5 μg ml^{-1}), erythromycin (Em, 1 μg ml^{-1}), kanamycin (Km, 25 μg ml^{-1}) or spectinomycin (Sp, 50 μg ml^{-1}) were added as appropriate. Solid medium was made with LB, SP or minimal C medium containing 1·5 % (w/v) Bacto Agar (Difco). To test for growth of *B. subtilis* integrant strains on L-arabinose as sole carbon source, strains were plated on minimal C medium containing 0·1 % (w/v) L-arabinose. The AraB$^-$ phenotype was determined on minimal C medium plates supplemented with 1 % (w/v) casein hydrolysate, 0·1 % L-arabinose and 1 % (w/v) ribitol. To determine specific growth rates, the *B. subtilis* strains were grown in liquid C medium with 0·4 % L-arabinose as sole carbon source. The cultures were incubated with aeration by shaking (130 r.p.m.) and cell growth was monitored by OD_{600}. For β-galactosidase assays and RNA preparation the *B. subtilis* strains were grown in liquid C medium supplemented with 1 % (w/v) casein hydrolysate, and L-arabinose and glucose were added to the cultures when necessary at a final concentration of 0·4 % (w/v).

DNA manipulations and sequencing. DNA manipulations were carried out according to Sambrook *et al.* (1989). Enzymes were purchased from commercial suppliers and used according to the manufacturers' instructions. DNA sequencing was performed by the method of Sanger *et al.* (1977) with the Sequenase Kit (T7 DNA polymerase; USB). Sequencing templates were prepared by a combination of subcloning appropriate fragments from pSNL1 and pSNL9 into the polycloning site of M13mp19 or M13mp18 (Yanisch-Perron *et al.*, 1985) and sequential deletion of the recombinant M13 derivatives, by the method of Dale *et al.* (1985), using the Cyclone Biosystem Kit (International Biotechnologies Inc.). The DNA sequence was determined on both strands and across all the restriction sites used for subcloning. The primer 5′ CCTCTTCGCTATTACGCC 3′, complementary to the coding sequence of *lacZ*, was used to sequence the transcriptional *lacZ* fusions.

Plasmid constructions. pSNL7 was constructed by subcloning a 959 bp *Sma*I–*Pst*I DNA fragment (nt 938–1897, Fig. 1) from pSNL1 (Sá-Nogueira & Lencastre, 1989) between the *Sma*I and *Pst*I sites of the integrational vector pJM783 (Perego, 1983). To construct pSS2, we digested pSNL1 (Sá-Nogueira & Lencastre, 1989) with *Hin*dIII and *Xho*I and cloned a purified fragment of 965 bp (nt 3815–4780, Fig. 1) between the *Hin*dIII and *Sal*I sites of the integrating vector pJH101 (Ferrari *et al.*, 1983). pTN10 was obtained by subcloning a 789 bp *Hin*dIII–*Hin*cII DNA fragment (nt 6545–7334, Fig. 1) from pSS3 between the *Hin*dIII and *Eco*RV sites of the integrational vector pJH101 (Ferrari *et al.*, 1983). pTN14 was constructed by subcloning the 678 bp *Sma*I–*Bgl*II DNA fragment (nt 8242–8920, Fig. 1) from pTN13 between the *Bam*HI and *Sst*I (fill-in) sites of pJM783 (Perego, 1993). pSNL10 was obtained by subcloning a 1·7 kb *Eco*RI–*Hin*cII fragment (nt 2681–4416, Fig. 1) from pSNL1 (Sá-Nogueira & Lencastre, 1989) between the *Eco*RI and *Sma*I sites of pMK4 (Sullivan *et al.*, 1984).

Table 1. *B. subtilis* strains

Strain*	Genotype	Phenotype	Source†
168T$^+$	Prototroph	Ara$^+$	F. E. Young
BR151	*metB10 lys3 trpC2*	Ara$^+$	F. E. Young
IQB100	*araB'*::pSNL7(*araB–cat lacZ*)	Cm^r Ara$^-$	pSNL7 → 168T$^+$
IQB101	*araB'*::*lacZ erm*	LacZ$^+$ Em^r Ara$^-$	pSNL11‡ → 168T$^+$
IQB102	*araB'*::*erm lacZ*	LacZ$^-$ Em^r Ara$^-$	pSNL12‡ → 168T$^+$
IQB103	*araA'*::pSNL13 (*araA–lacZ cat*)	LacZ$^+$ Cm^r Ara$^-$	pSNL13 → 168T$^+$
IQB104	*araA'*::pSNL14 (*araA–cat lacZ*)	LacZ$^-$ Cm^r Ara$^+$	pSNL14 → 168T$^+$
IQB202	*araL'*::pSS2 (*araL–amp cat*)	Cm^r Ara$^+$	pSS2 → 168T$^+$
IQB204	*araN'*::pTN10 (*araN–cat amp*)	Cm^r Ara$^+$	pTN10 → 168T$^+$
IQB205	*araQ'*::pTN14 (*araQ–lacZ 'cat*)	LacZ$^-$ Cm^r Ara$^+$	pTN14 → 168T$^+$
IQB206	Δ*araL–abfA*::*spc*	Sp^r Ara$^+$	pSN22‡ → 168T$^+$

* All strains are derivatives of *B. subtilis* 168T$^+$.

† The arrows indicate transformation and point from donor DNA to recipient strain. F. E. Young, University of Rochester New York, USA.

‡ Transformation was carried out with linearized plasmid DNA.

pSNL11 and pSNL12 were obtained as follows. A 4·5 kb *Bam*HI–*Hin*dIII (fill-in) fragment extracted from pMC11 (Debarbouillé *et al.*, 1990), containing *lacZ* and *erm* from pTV32 (Perkins & Youngman, 1986), was subcloned in both orientations at the unique *Eco*RV restriction site (nt 3214, Fig. 1) of pSNL10. pSNL13 and pSNL14 were obtained by subcloning a 470 bp *Dra*I–*Eco*RV DNA fragment (nt 82–552, Fig. 1) from pSNL9 at the unique *Sma*I site of the integrational vector pJM783 (Perego, 1993) in both orientations. pSNL13 contains *lacZ* in the same orientation as the *araA* region sequences and pSNL14 contains *lacZ* in the opposite orientation. pSN20 was constructed by cloning the 1·2 kb *Eco*RV–*Hin*cII fragment (nt 3214–4416, Fig. 1) from pSS3 into the *Sma*I site of pAH248 [a pGem-7Zf(+) (Promega) derivative that contains a Km^r gene cloned between its *Xho*I and *Eco*RI sites (A. O. Henriques & C. P. Moran Jr, Emory University School of Medicine, Atlanta, GA, USA, personal communication)]. To obtain pSN21 a 1·7 kb *Eco*RV fragment from pSN5 (nt 10632–about 12332, Fig. 1) was inserted into the *Hin*cII site of pAH250 [a pBluescript SK+ (Stratagene) derivative that contains a Sp^r gene (*spc*) cloned into the *Eco*RV site (A. O. Henriques, B. W. Beall & C. P. Moran Jr, personal communication)]. To construct pSN22, we digested pSN20 with *Pst*I and *Nsi*I and cloned a purified fragment of about 2790 bp, which contains the Km^r gene, in the *Sma*I site of pSN21. pSNL9, pSS3, pTN13 and pSN5 were obtained by cutting chromosomal DNA from *B. subtilis* strains IQB100, IQB202, IQB204 and IQB205 (Table 1) with *Hin*dIII, *Eco*RI, *Nco*I and *Sma*I, respectively, followed by circularization of the DNA fragments at low concentration.

Bacterial transformation. *B. subtilis* DNA transformations were performed according to the method of Anagnostopoulos & Spizizen (1961). *E. coli* transformations were carried out according to standard methods (Sambrook *et al.*, 1989).

β-Galactosidase assays. Strains of *B. subtilis* harbouring transcriptional *lacZ* fusions were grown in 75 ml C medium supplemented with 1% casein hydrolysate. During early exponential phase (OD_{600} = 0·11–0·15) 25 ml of the culture was transferred to two different flasks and L-arabinose at a final concentration of 0·4% or both L-arabinose and glucose each at a final concentration of 0·4% were added. At this time, t_0, 100 µl aliquots of cell culture were collected, harvested and stored at −70 °C overnight. Exponential growth of the three cultures was followed by measuring OD_{600} and at 30 min intervals, 100 µl of cell culture samples was removed and stored at −70 °C until the cultures reached an OD_{600} = 0·7–0·8, which corresponds to growth for at least 2·5 generations in the presence of the inducer. The cells were resuspended in 1 ml Z buffer (Miller, 1972) and two drops of chloroform plus one drop of 0·1% SDS were added and mixed vigorously for 10 s on a table top vortex apparatus. β-Galactosidase activity was determined as described by Miller (1972) using the substrate ONPG.

RNA preparation, Northern blotting and primer extension analysis. *B. subtilis* 151 or 168T$^+$ cells were grown in C medium supplemented with 1% casein hydrolysate in the presence and absence of L-arabinose at a final concentration of 0·4%. Cells were harvested during late exponential phase (OD_{600} ~ 0·9) and RNA prepared as described by Igo & Losick (1986). For Northern blot analysis, 2·5–10 µg total RNA was run in 1·0–1·2% (w/v) agarose/formaldehyde and transferred to positively charged nylon membranes (Hybond-N+, Amersham) according to standard methods (Sambrook *et al.*, 1989). Size determination was done using an RNA ladder (0·24–9·5 kb; Gibco/BRL). The probes were labelled using the Multiprime random-prime DNA labelling system from Amersham and [α-^{32}P]dATP [3000 Ci mmol^{-1} (111 TBq mmol^{-1})]. Primer extension analysis was performed as described by Sambrook *et al.* (1989). The two synthetic oligonucleotides used in primer extension experiments were primer A (5′ GAAGCATGTAAACTGCCCC 3′), complementary to nt 216–234 (Fig. 1), and primer B (5′ CCAGCGTCTCTTCCCCG 3′), complementary to nt 283–300 (Fig. 1). The two oligonucleotides were used in separate experiments to rule out the possibility of primer-specific artifacts. A total of 10 ng of primer was used in the labelling reaction mixed with 25 µg RNA, denatured by heating to 85 °C for 10 min and annealed by incubation at 42 °C for 3 h. The oligonucleotide primer was extended using 15 units of avian myeloblastosis virus reverse transcriptase for 2 h at 37 °C, as described by

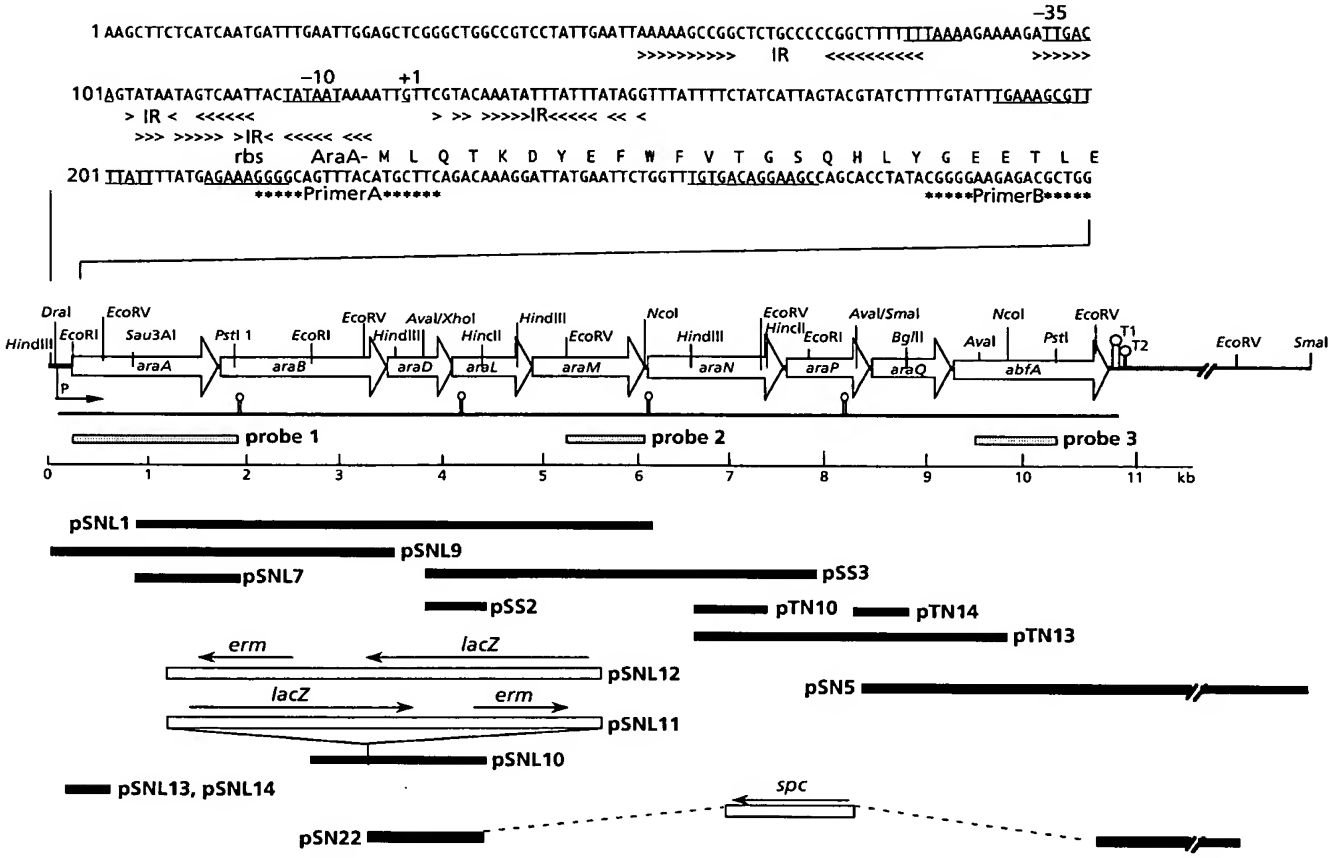


Fig. 1. Physical and genetic map of the *ara* region of the chromosome. The location and direction of transcription of the nine ORFs (*araA, B, D, L, M, N, P, Q* and *abfA*), predicted from the analysis of the nucleotide sequence, are indicated by arrows. The promoter (P) of the *ara* operon, defined by primer extension, is located upstream from *araA* and the two regions of dyad symmetry (T1 and T2) that could represent the terminators of the *ara* transcriptional unit are located downstream from *abfA*. Relevant restriction sites are given in the partial restriction map. The region to the right of the *Eco*RI site (position 11755) is not drawn to scale. Immediately below the physical map the *ara* operon transcript is schematically shown and putative secondary structures of the mRNA are indicated by stem–loop structures. The grey boxes, below the physical map, represent the three fragments used as probes for Northern analysis of the *ara* transcripts and the black boxes represent the extent of the inserts in the indicated plasmids. The sites of different insertion-deletion mutations resulting from replacement of wild-type sequences, by double cross-over events (confirmed by Southern blot analysis, data not shown), with *in vitro*-engineered fragments of the *ara* region, present in plasmids pSNL11, pSNL12 and pSN22, are also shown. Plasmids pSNL7, pSS2, pTN10, pTN14, pSNL13 and pSNL14 were integrated into the host chromosome by means of a single cross-over (Campbell-type) recombinational event that occurred in the region of homology (confirmed by Southern blot analysis, data not shown). The *ara* operon promoter nucleotide sequence of the non-transcribed strand is shown in the 5′–3′ direction above the physical map. The predicted N-terminal region of the polypeptide encoded by *araA* is given in single letter code. The transcription start site (+1), defined by primer extension analysis, the −35 and −10 regions of the promoter and the putative ribosome binding site (rbs) are underlined. Convergent arrows represent different regions of dyad symmetry (IR) and the complementary sequence of the two primers A and B, used in primer extension analysis are represented below the sequence. The two putative catabolic-repression-associated sequences (positions 191–204 and 260–273) are underlined.

Sambrook *et al.* (1989). Analysis of the extended products was carried out on 7·5% polyacrylamide urea gels.

Computer analysis. Amino acid sequences were deduced from the nucleotide sequence using DNASIS V2.0 (Hitachi Software Engineering, 1991). The GenBank and EMBL databases were accessed using the GCG package of sequence analysis software (Genetics Computer Group, Madison, Wisconsin, USA).

RESULTS

Insertional inactivation of *araB* and cloning of an intact copy of *araA*

The location of the *araA* locus at one end of the cloned fragment in pSNL1 (Fig. 1), together with the absence of *araA* complementation with pSNL1, suggested that only

Table 2. Percentage amino acid identity between the predicted sequences of the Ara proteins and similar proteins

B. subtilis AraA protein	Homologue (species/accession no.)*	Function	Identity (%)	Amino acid overlap
AraA	AraA (*E. coli*/M15263)	L-Arabinose isomerase	52·9	495
	AraA (*Sal. typhimurium*/M11047)	L-Arabinose isomerase	52·9	495
AraB	AraB (*E. coli*/M15263)	L-Ribulokinase	25·7	552
	AraB (*Sal. typhimurium*/M11045)	L-Ribulokinase	30·6	350
AraD	AraD (*E. coli*/M15263)	L-Ribulose-5-P 4-epimerase	57·1	231
	AraD (*Sal. typhimurium*/M11046)	L-Ribulose-5-P 4-epimerase	58·0	205
AraL	NagD (*E. coli*/X14135)	Unknown	25·5	251
AraN	LacE (*Agrobacterium radiobacter*/X66596)	Lactose-binding protein	26·2	302
	MalX (*Streptococcus pneumoniae*/L08611)	Maltose-binding protein	24·1	345
	AmyE (*Thermoanaerobacterium thermosulfurigens*/M57692)	Starch-binding protein	21·7	369
AraP	LacF (*Agrobacterium radiobacter*/X66596)	Membrane protein	29·6	284
	UgpA (*E. coli*/X13141)	Membrane protein	26·2	286
	AmyD (*Thermoanaerobacterium thermosulfurigens*/M57692)	Membrane protein	25·4	284
	MalC (*Streptococcus pneumoniae*/L08611)	Membrane protein	25·2	298
AraQ	LacG (*Agrobacterium radiobacter*/X66596)	Membrane protein	32·7	254
	UgpE (*E. coli*/X13141)	Membrane protein	22·9	279
	AmyC (*Thermoanaerobacterium thermosulfurigens*/M57692)	Membrane protein	28·2	262
	MalD (*Streptococcus pneumoniae*/L08611)	Membrane protein	25·6	262
AbfA	AbfA (*Streptomyces lividans*/U04630)	α-L-Arabinofuranosidase	52·6	500

part of *araA* was present in this plasmid (Sá-Nogueira & Lencastre, 1989). To clone the entire *araA* gene, plasmid pSNL7 (Fig. 1) was integrated, as single copy, into the *B. subtilis* 168T$^+$ chromosome at the *araA* and *araB* region of homology. This procedure causes disruption of the transcriptional unit and the structure of the resulting strain IQB100 that was unable to grow on minimal medium containing L-arabinose as sole carbon source, confirming the polar effect of the insertion on the genes located downstream from *araA*. Furthermore, strain IQB100 showed resistance to ribitol in the presence of L-arabinose on minimal medium plates supplemented with 1% casein hydrolysate. In *B. subtilis* (Paveia & Archer, 1992a), like in *E. coli* (Katz, 1970), these results indicate a defective *araB*. Chromosomal DNA from IQB100 was used to rescue the entire *araA* gene and its upstream region (see Methods). The structure of the recircularized plasmid, pSNL9, was analysed and it contains a 950 bp fragment of DNA upstream from the previously cloned DNA in plasmid pSNL7 (Fig. 1).

Cloning of the chromosomal region extending downstream from *araD*

To clone the region located downstream from *araD*, an integrational plasmid, pSS2, carrying sequences of *araD* and *araL* (Fig. 1), was transformed into the wild-type strain 168T$^+$. After integration as single copy, the resulting strain IQB202 presented an Ara$^+$ phenotype although the growth on minimal medium plates with L-arabinose as sole carbon source was slower than that observed with the wild-type strain 168T$^+$ (see Discussion below). The digestion of total chromosomal DNA from IQB202 followed by circularization of the fragments yielded plasmid pSS3 that includes a 3·0 kb insert located downstream to the fragment cloned in pSS2 (Fig. 1). To obtain a fragment that would contain the downstream region from *araN*, we performed a second chromosome walking step, using integrational plasmid pTN10 (Fig. 1). This procedure created plasmid pTN13 that carried an additional 3·2 kb of DNA adjacent to the previously cloned fragment in plasmid pTN10 (Fig. 1). Strain IQB204, which resulted from the integration of plasmid pTN10 (Fig. 1) into the chromosome of the wild-type strain 168T$^+$ showed a Ara$^+$ phenotype similar to that seen with IQB202. A third chromosome walking step rightwards from pTN13, using integrational plasmid pTN14 (Fig. 1), isolated a 4·7 kb *Sma*I fragment (plasmid pSN5). Plasmid pTN14, when integrated into the chromosome of strain IQB205 as single copy, caused an Ara$^+$ phenotype. The structure of the inserts in pSS3, pTN13 and pSN5 was compared to that of the corresponding areas of chromosomal DNA by Southern blot analysis (data not shown) and the results revealed that no detectable rearrangement occurred during the cloning process.

DNA sequence and deduced products of *ara* genes

Appropriate restriction fragments, selected on the basis of the physical maps of pSNL1, pSNL9, pSS3, pTN13 and pSN5, were subcloned into M13mp18 and M13mp19 and used as templates to determine the nucleotide sequence of the 11 kb DNA region shown in

Fig. 1. Sequence analysis revealed the presence of nine ORFs; the first three, by their position in the sequenced fragments of pSNL1 and pSNL9 and according to our previous results (Sá-Nogueira & Lencastre, 1989), were identified as *araA*, *araB* and *araD* (Fig. 1). *araA*, *araB* and *araD* could encode 496, 560 and 229 aa products of 56·2, 60·9 and 25·7 kDa, respectively. The six ORFs found downstream from *araD*, here named *araL*, *M*, *N*, *P*, *Q* and *abfA* (Fig. 1), of 269, 394, 433, 313, 281 and 499 codons, are capable of encoding putative products of 29, 43·1, 48·7, 35, 31·8 and 57 kDa, respectively. All ORFs are preceded by strong ribosome binding sites with the exception of *araL* which possesses a weak ribosome binding site. The intercistronic regions are very short and overlaps were observed between the *araD* and *araL* coding sequences, and between *araL* and *araM*, suggesting translational coupling. Two potential hairpin–loop structures, situated next to the UAA stop codon of *abfA* (T_1 and T_2, Fig. 1, with ΔG values of $-27{\cdot}4$ and $-18{\cdot}7$ kcal mol^{-1}, respectively, according to Tinoco *et al.*, 1973), probably correspond to transcription terminators. The absence of transcriptional signals among the nine coding regions suggested that they form a large operon transcribed from a promoter (described below) positioned 104 nt upstream from the *araA* start codon (Fig. 1).

Comparison of the primary structures of the products predicted to be encoded by the *ara* genes with GenBank sequences revealed significant similarities with other bacterial proteins of known function and the results are summarized in Table 2. The putative product of *araM*, a hydrophilic protein, did not show any significant similarity. The *araA*, *araB* and *araD* gene products exhibited a high level of identity to the L-arabinose isomerase, L-ribolukinase and L-ribulose-5-phosphate 4-epimerase, respectively, of *E. coli* and *Salmonella typhimurium*. The product of *araL*, a hydrophilic protein, displayed similarity to the *nagD* gene product of unknown function, which belongs to the *nag* regulon of *E. coli* involved in the metabolism of *N*-acetyl glucosamine (Plumbridge, 1989). The N-terminal region of the predicted sequence also shared 28·1% and 29·2% identity (over 121 and 106 aa, respectively, data not shown) with two 4-nitrophenylphosphatases, Pho2 and Pho13, from *Schizosaccharomyces pombe* (Yang *et al.*, 1991) and *Saccharomyces cerevisiae* (Kaneko *et al.*, 1989), respectively.

The predicted primary structure of *araN* showed similarity to known sugar-binding proteins that belong to the family of binding-protein-dependent transport systems (Table 2). Although the identity was not very high, there was significant sequence conservation within the N-terminal region of these proteins which display a signature sequence, according to Tam & Saier (1993). On the basis of this signature sequence (Fig. 2a) AraN can be included in the cluster 1 binding proteins (according to Tam & Saier, 1993), together with the above-mentioned proteins involved in the transport of malto-oligosaccharides and multiple sugars. The hydropathy profile of AraN indicated that it is mainly a

(a)

```
Signature sequence  LXXLGKXFEXDXXGIKVXV  (68-81)
                    I  IAD YT E  NV I L
                    V  VIQ  N A  DY   P
                    A  WV        P
            AraN    YVEMVKEWNKKYPDRKIKLNTVVYPY  (75)
```

(b)

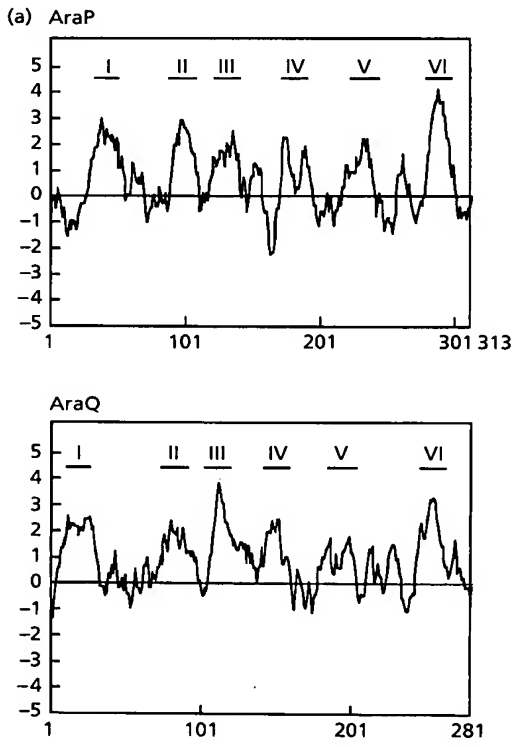
```
                    10        20        30        40
Abf-B. su   MKKARMIVDKEYKIGEVDKRIYGSFIEHMGRAVYEGIYEPDHPEADEDG
            ::: ::..:..::.:.:::::::::::::::::::::::::::.:.:.:
Abf-B. st   ATKKATMIIEKDFKIAEIDKRIYGSFIEHLGRAVYGGIYEPGHPQADENG
                    10        20        30        40        50
```

Fig. 2. (a) Alignment of a segment of the predicted sequence of the AraN protein with the signature sequence of cluster 1 binding proteins, from binding-protein-dependent transport systems, according to Tam & Saier (1993). Numbers in parentheses indicate the positions of the last amino acid residues. The highly conserved lysine residue (K) is in bold and the amino acid residues that match the signature sequence are underlined. (b) Alignment of the N-terminal sequence (deduced from the nucleotide sequence) of AbfA from *B. subtilis* (B. su) with the N-terminal sequence of α-L-arabinofuranosidase from *B. stearothermophilus* (B. st). Double dots represent identical amino acids and single dots represent conservative changes.

hydrophilic protein; however its N-terminal region displayed characteristics of signal peptides of secretory precursor proteins: a positively charged N terminus, a hydrophobic core and a sequence, IAGCSA (starting at aa 19), which corresponds to the consensus sequence for the precursors of lipoproteins (reviewed in Hayashi & Wu, 1990).

The predicted products of *araP* and *araQ* exhibited hydropathy profiles (according to Kyte & Doolittle, 1982) characteristic of integral membrane proteins: six major regions of high hydrophobicity (hydropathic index $> 1{\cdot}0$), each composed of at least 20 aa which could be capable of spanning the membrane (Fig. 3a). AraP and AraQ shared an identity of 19·6% and showed significant similarity with integral cytoplasmic membrane proteins involved in prokaryotic binding-protein-dependent transport systems (Table 2). In common with most of these integral membrane proteins, AraP and AraQ have a conserved hydrophilic segment (Fig. 3b) at approximately 100 residues from the C terminus with the consensus EAA---G---------I-LP (Dassa & Hofnung, 1985). Furthermore, on the basis of this signature sequence, they can be included in the disaccharide subcluster proposed by Saurin *et al.* (1994) together with the above-mentioned proteins involved in the transport of malto-oligosaccharides, multiple sugars and α-glycerol phosphate.

The deduced product of *abfA*, a hydrophilic protein, displays a N-terminal region (Fig. 2b) which resembles a signal peptide of exoproteins (reviewed on Gierasch, 1989 and Nagarajan, 1993): a positively charged N terminus, a hydrophobic core and a potential cleavage site (AV, position 32–33, Fig. 2b). The primary structure of the putative product of *abfA* is closely related to the



(b)

```
AraP      198 GLQNVPKELYEAADIDGANTMKKFLHITLPFLKPVTVYVL (109)
              .:::.:..: :::.:::::::::: .:.:::.::: . .:
Consensus     ALQSIPDSLIEAAKIDGAGPFQRFWNIVLPLLKPVLAVLL
              ..: .:...:..:: . : :..:. ::.:::.....
AraQ      165 YALGLPRDLLDSARMDGCTEFGIFFRIMAPLMKPAFGAMI (100)
```

Fig. 3. (a) Hydropathic index for the deduced amino acid sequences of AraP and AraQ according to the algorithm of Kyte & Doolittle (1982). The hydropathy profiles are plotted from the N terminus to the C terminus by averaging hydropathy values over a window of 10 residues. Hydrophobic segments which could correspond to membrane-spanning regions are labelled I–VI. (b) Alignment of a hydrophilic segment, at approximately 100 residues from the C terminus of the predicted sequences of AraP and AraQ, with the consensus sequence for the group of integral cytoplasmic membrane proteins from binding-protein-dependent transport systems (Saurin *et al.*, 1994), which includes permeases involved in the transport of disaccharides and glycerol phosphate. The general consensus for integral membrane proteins from binding-protein-dependent permeases, EAA—G---------I-LP, where (-) represents any amino acid (Dassa & Hofnung, 1985), is underlined. The distance of the invariant glycine residue from the C terminus is represented in parentheses. Double dots represent identical amino acids and single dots represent conservative changes.

α-L-arabinofuranosidase of *Streptomyces lividans* (Table 2) and the N-terminal region (Fig. 2b) is 74% identical and 96% similar to the sequenced N terminus of purified α-L-arabinofuranosidase from *Bacillus stearothermophilus* (Gilead & Shoham, 1995). These observations strongly suggest that *abfA* encodes an α-L-arabinofuranosidase.

RNA transcript analysis of the L-arabinose gene region

Total RNA from cells grown in the presence and absence of L-arabinose was isolated, blotted and hybridized to three different DNA probes (probes 1, 2 and 3, Fig. 1) each specific to one gene of the *ara* region (*araA*, *araM* and *abfA*, respectively). Northern blot analysis (Fig. 4) revealed that *ara* genes are organized in a large polycistronic operon, and that transcripts could be detected only if the cells were grown in the presence of L-arabinose. In addition to a transcript of 11 kb comprising all genes and detected with the three probes, several other signals of different intensities were obtained depending on the probe used (Fig. 4). Using the *araA*-specific probe, we detected five different transcripts of about 8·2, 6·4, 5·8, 4 and 1·9 kb, considering a margin of error of 10–15% for the size determination of transcripts. Two additional transcripts of about 8·2 and 6·4 kb were visualized with the *araM*-specific probe and three hybridization signals were obtained with the *abfA*-specific probe: 8·3, 4·8 and 1·1 kb. Interestingly, stable secondary structures were identified at the corresponding sites within the *araB*, *araL*, *araN* and *araQ* sequences (Fig. 1). The exact nature of these different minor transcripts is unknown but they might be generated by premature transcription termination and/or processing of the multicistronic messenger or RNA degradation. Another possible explanation is the presence of transcription initiation sites located downstream from the promoter defined by primer extension analysis (see below).

The promoter region and transcriptional start site of the *ara* operon

To determine the transcriptional start site of the *ara* operon, total RNA was extracted during the exponential growth of wild-type cultures in the presence and in the absence of L-arabinose. Reverse transcripts were obtained using an end-labelled 17-mer (primer B, Fig. 1), designed to hybridize to part of the *araA* mRNA. A single extension product was detected with RNA isolated from cells grown in the presence of L-arabinose, the size of which suggests that transcription of the *ara* operon starts at a G residue situated 97 nt upstream from the *araA* start codon (Fig. 5). No extension product was seen when RNA was isolated from cells grown in the absence of L-arabinose. The same transcription start point was obtained using a second primer (primer A, Fig. 1) designed to hybridize to part of the mRNA 50 bases upstream from the first primer (Fig. 5). The synthesis of the *ara* operon mRNA is induced by L-arabinose and driven by a strong promoter as evaluated by the intensity of the reverse transcript signal obtained. Situated 7 and 30 bp upstream from the *ara* operon transcription start site are sequences identical to the consensus −35 and −10 regions (TTGACA-17 bp-TATAAT), respectively, of promoters recognized by *B. subtilis* σ^A-containing RNA polymerase (Moran *et al.*, 1982). Sequence analysis of the promoter region revealed the existence of three inverted repeats, putative

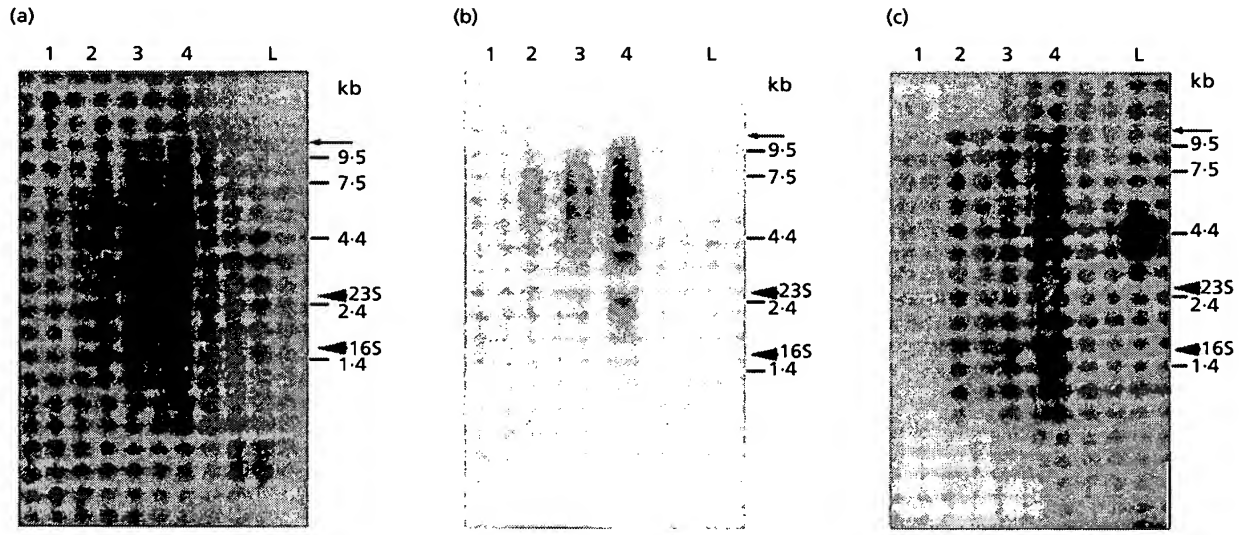


Fig. 4. Northern analysis of the *ara* operon-specific transcripts. Lanes: 1, 10 μg total RNA extracted from the uninduced wild-type strain *B. subtilis* 168T$^+$; 2, 3 and 4, 2·5 μg, 5 μg and 10 μg, respectively, of total RNA extracted from the induced wild-type strain *B. subtilis* 168T$^+$ grown on L-arabinose (see Methods); L, 4 μg RNA ladder (0·24–9·5 kb; Gibco/BRL). The samples were run in 1% (a, b) and 1·2% (c) agarose formaldehyde denaturing gel. The ^{32}P-labelled probes used were synthesized from (a) a 1·6 kb *Eco*RI–*Pst*I fragment (position 249–1897, probe 1), (b) a 0·8 kb *Nco*I–*Eco*RV fragment (position 5270–6079, probe 2) and (c) a 0·7 kb *Pst*I–*Ava*I fragment (position 9538–10275, probe 3). The RNA ladder was probed with ^{32}P-labelled λ DNA and also visualized by staining with ethidium bromide. The transcript of about 11 kb comprising all genes and detected with the three probes is indicated by an arrow.

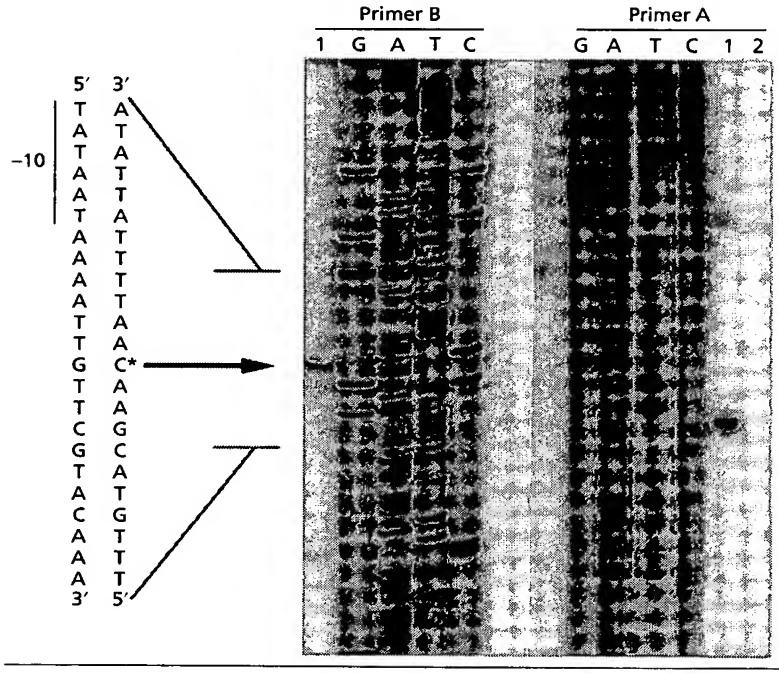


Fig. 5. Primer extension analysis of the *ara* operon promoter. Two radiolabelled oligonucleotide primers, A and B, complementary to two different regions downstream from the *araABD* promoter [primer A, 5′ GAAGCATGTAAACTGCCCC 3′, complementary to a region of *araA* mRNA located between nucleotides 216 and 234 (Fig. 1) and B, 5′ CCAGCGTCTCTTCCCCG 3′, complementary to a region of the *araA* mRNA located between nucleotides 283 and 300 (Fig. 1)] were hybridized with *B. subtilis* BR151 RNA isolated from exponentially growing cells in the presence (lane 1) or absence (lane 2) of L-arabinose. After extension, the products were analysed by gel electrophoresis, together with a set of dideoxynucleotide chain-termination sequencing reactions using the same primers and a single-stranded M13 DNA template which includes the entire *araA* gene and an additional 228 bp of its 5′ flanking sequence.

operator-like sequences, in the −35 and −10 regions (Fig. 1). A potential hairpin–loop structure with a Δ*G* value of −19·2 kcal mol^{-1} (Tinoco *et al.*, 1973), centred 27 bp upstream from the −35 region (Fig. 1), probably corresponds to a transcription terminator of a gene located upstream from the cloned DNA fragment.

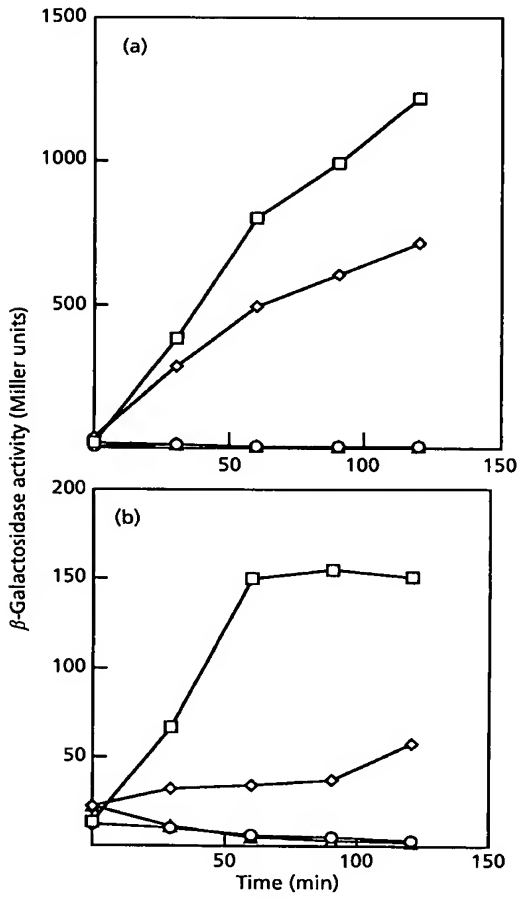


Fig. 6. Expression of the *ara* operon measured by determination of the levels of β-galactosidase activity (Miller units) present in exponentially growing cells. Strains of *B. subtilis* harbouring transcriptional *lacZ* fusions were grown on minimal C medium supplemented with 1% casein hydrolysate and either (a) 0·4% L-arabinose or (b) 0·4% L-arabinose plus 0·4% glucose (see Methods). Time is expressed in minutes after induction. ◇, IQB101 (*araB'–lacZ erm*; Ara⁻ Em^r LacZ⁺); □, IQB103 (*araA'–lacZ cat*; Ara⁺ Cm^r LacZ⁺); △, IQB102 (*araB'–erm lacZ*; Ara⁻ Em^r LacZ⁻; negative control); ○, IQB104 (*araA'–cat lacZ*; Ara⁺ Cm^r LacZ⁻; negative control). For each strain the results represent the mean, in Miller units, of two independent experiments.

Expression of the *ara* operon is induced by L-arabinose and repressed by glucose

To study the regulation of expression of the operon we constructed transcriptional *lacZ* fusions at this locus. The replicative plasmids pSNL11 and pSNL12, carrying *lacZ* and *erm* (Fig. 1), were linearized and used separately to transform the wild-type 168T⁺ strain. This resulted in the integration of *lacZ* and *erm* into the chromosome at the *araB* locus. The resulting strains, IQB101 (*araB'–lacZ erm*) and IQB102 (*araB'–erm lacZ*), were unable to grow on L-arabinose as sole carbon source, which confirmed the insertional inactivation of *araB*. The integrational plasmids pSNL13 and pSNL14, carrying the same DNA fragment in opposite orientations (Fig. 1), were integrated as single copy into the chromosome of the wild-type strain 168T⁺. The resulting strains, IQB103 (*araA'–lacZ cat*) and IQB104 (*araA'–cat lacZ*), respectively, displayed an Ara⁺ phenotype because the integration was not disruptive. The LacZ phenotype of the four strains was tested on minimal C medium plates supplemented with 1% casein hydrolysate and X-Gal. Upon addition of L-arabinose to the medium, strains IQB101 and IQB103 presented a dark blue phenotype, whereas those of IQB102 and IQB104 remained white, confirming that the expression of the operon is driven from a promoter located upstream from *araA* and induced by L-arabinose. Furthermore, addition of other pentoses such as D-xylose and D-ribose failed to induce a LacZ⁺ phenotype in strain IQB103. The regulation of *ara* operon expression was examined in cultures during mid-exponential phase in minimal C medium supplemented with 1% casein hydrolysate as described in Methods. The levels and patterns of *lacZ* expression in IQB101 (*araB'–lacZ erm*; Ara⁻ LacZ⁺), IQB103 (*araA'–lacZ cat*; Ara⁺ LacZ⁺), IQB102 (*araB'–erm lacZ*; Ara⁻ LacZ⁻; negative control) and IQB104 (*araA'–cat lacZ*; Ara⁺ LacZ⁻; negative control) determined in the presence of L-arabinose and L-arabinose plus glucose are shown in Fig. 6. When the four strains were grown in the absence of inducer, the level of accumulated β-galactosidase activity, at time $t = 120$ min, was 4·4, 4·8, 2·8 and 1·8 Miller units, respectively. In the presence of L-arabinose the pattern of expression observed in strains IQB101 (*araB'–lacZ*; Ara⁻) and IQB103 (*araA'–lacZ*; Ara⁺) was very similar (Fig. 6) but the levels of accumulated β-galactosidase activity in the *araB* null mutant were less than 60% relative to the wild-type strain (discussed below). Addition of glucose reduced the level of expression to less than 12% in both Ara⁺ and Ara⁻ backgrounds (Fig. 6). These data demonstrate that L-arabinose is an inducer which stimulates the expression of the *ara* operon at the transcriptional level and transcription is subjected to catabolite repression by glucose. Furthermore, the prediction that the expression of the *ara* operon is driven from a strong promoter, made on the basis of the intensity of the reverse transcript signal observed in primer extension analysis, was confirmed when β-galactosidase activity was measured in strain IQB103 (*araA'–cat lacZ*; Ara⁺).

araL, M, N, P, Q and *abfA* are not required for L-arabinose utilization

Strains IQB202 and IQB204 in which the integration of plasmids pSS2 and pTN10, respectively, interrupted the transcription unit at *araL* and *araN* (Fig. 1), exhibited an Ara⁺ phenotype, however, their growth on minimal medium plates with L-arabinose as sole carbon source was slightly slower than the one observed with the wild-type strain 168T⁺. This phenotype was not observed with strain IQB205 in which pTN13 disrupted the operon at the end of *araQ*. To confirm that *araL*, *M*, *N*,

P, *Q* and *abfA* are not required for L-arabinose utilization, we constructed a deletion in the region downstream from *araD* by replacing *in vitro* the wild-type sequences of *araL*, *M*, *N*, *P*, *Q* and *abfA* with a Sp^r cassette and then using it to replace the corresponding chromosomal sequences (see Methods). Plasmid pSN22 (Fig. 1) was linearized and used to transform the wild-type strain $168T^+$ Sp^r. The resulting strain IQB206, was Km^s which indicated that the Sp^r phenotype was the result of a double cross-over event that occurred on both sides of the cassette inserted between the *araL* and *abfA* sequences (Fig. 1). This mutant strain was able to grow on minimal medium plates with L-arabinose but displayed a phenotype even more drastic than the one exhibited with strains IQB202 and IQB204. To quantify this observation we determined the specific growth rate of the deletion-insertion mutant and the wild-type strain in liquid minimal C medium with L-arabinose as sole carbon source, as described in Methods. The doubling time of strain IQB206 was 1·8-fold higher than the wild-type strain $168T^+$, $193{\cdot}4 \pm 7{\cdot}2$ and $107{\cdot}7 \pm 3{\cdot}6$ min (means of three independent experiments ± SEM), respectively. These results confirmed that the genes located downstream from *araD* in the operon are not essential for L-arabinose utilization, however their absence in the deletion mutant affects the specific growth rate in minimal medium with L-arabinose as the sole carbon source when compared to the wild-type strain.

DISCUSSION

In this study we have described a new catabolic operon involved in the utilization of L-arabinose in *B. subtilis*, which we designated *ara*. The arabinose metabolic genes *araA*, *araB* and *araD*, encoding L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively, were cloned previously and by complementation experiments the products of *araB* and *araD* were shown to be functionally homologous to their *E. coli* counterparts (Sá-Nogueira & Lencastre, 1989). These genes, whose inactivation leads to an Ara^- phenotype, were found to be the first three ORFs of a nine cistron transcriptional unit whose total length is 11 kb. To our knowledge this operon is the largest catabolic operon described in *B. subtilis*. As expected from the occurrence of genetic complementation, the deduced products of *araA*, *araB* and *araD* from *B. subtilis* display a very high level of identity to the corresponding enzymes from *E. coli* and *Sal. typhimurium*, which indicates that this metabolic pathway was fundamentally conserved during evolution. In *B. subtilis* the metabolic gene order, *araABD*, coincides with the order of the enzymic steps carried out by the proteins they encode. This order is different from the one found in the operons of the *Enterobacteriaceae* members *E. coli* and *Sal. typhimurium*, *araBAD*, so it seems that the three genes did not act as a unitary block in the evolution of the eubacterial *ara* genes.

The six ORFs found downstream from *araD*, here named *araL*, *M*, *N*, *P*, *Q* and *abfA*, are not required for L-arabinose utilization. This was shown in a mutant strain, IQB206, bearing a deletion in the region downstream from *araD* comprising all genes. The function of *araL* and *araM* is unknown. The putative product of *araM* did not show any significant similarity with other bacterial proteins of known function and the weak similarities displayed by *araL* did not suggest any particular function. Interestingly, the N-terminal sequence of *araL* shares an identity of 18·7% over 193 residues with the C-terminal sequences of *araM* (data not shown). The primary sequences of the products of *araN*, *araP* and *araQ* strongly suggest that they have a similar function to that of a superfamily of membrane-bound nutrient transport systems (Higgins *et al.*, 1990). Sequence similarities to known import proteins and the organization of the genes in the operon revealed the presence of three components of these transport systems. Firstly, the N terminus of AraN has a predicted signal peptide and sequences typical of Gram-positive lipoproteins (IAGCSA, starting at aa 19). We therefore suggest that AraN might be anchored in the cytoplasmic membrane via an amino-lipid group (Gilson *et al.*, 1988; Perego *et al.*, 1991). Secondly, *araP* and *araQ* gene products, as other characterized integral cytoplasmic membrane proteins, have hydropathy profiles which are virtually superimposable and some of their residues are apparently conserved (Fig. 3). Finally, *araN*, *araP* and *araQ* belong to the same operon and the ligand-specific binding protein, AraN, is encoded by the promoter-proximal gene, a situation common to these systems. In *B. subtilis* the phosphotransferase system is not involved in the transport of L-arabinose into the cell (Gay *et al.*, 1973). Therefore, it is tempting to propose that AraN, AraP and AraQ are components of a high affinity transport system for L-arabinose. However, no evident ATP-binding protein connected with energy coupling of the transport system was found in the operon.

The transport of L-arabinose across the *E. coli* cytoplasmic membrane requires the expression of either the high-affinity transport operon, *araFGH*, a binding-protein-dependent system (Horazdovsky & Hogg, 1989; Kolodrubetz & Schleif, 1981) or the low-affinity transport operon, *araE*, a proton symporter (Novotny & Englesberg, 1966). The existence of two parallel uptake systems thwarts usual genetic attempts to isolate mutants defective in either of the transport systems. The Ara^+ phenotype displayed by the *B. subtilis* deletion-insertion mutant strain IQB206 (*Δara–abfA::spc*) together with the 1.8-fold increase in doubling time observed on liquid minimal medium with L-arabinose as the sole carbon source, relative to the wild-type strain, is typical of a transport mutant when the micro-organism has alternative transport systems for the same substrate. An additional explanation for this phenotype observed in the deletion-insertion mutant is that insertion of *spc* might result in a less stable mRNA encoding *araABD*, leading to decreased amounts of their products. Interestingly, the primary structure of AraP and AraQ showed weak similarity with AraH, the integral cytoplasmic membrane protein from *E. coli*, and the same result was observed between AraN and AraF, the *E. coli*

arabinose binding protein (data not shown). Furthermore, on the basis of their signature sequences, AraN, AraP and AraQ can be included in the disaccharide subcluster (Figs 2 and 3) together with proteins involved in the high-affinity transport of malto-oligosaccharides and multiple sugars. *B. subtilis* secretes three enzymes involved in the degradation of L-arabinose polymers, an endo-arabanase and two arabinosidases, and the purified endo-arabanase has been shown to be capable of releasing arabinosyl oligomers from plant cell walls (Kaji & Saheki, 1975; Weinstein & Albersheim, 1979). To account for these observations a wider substrate range, L-arabinose and/or L-arabinose oligomers, for the *B. subtilis* AraN binding protein is suggested. The last gene of the *ara* operon, *abfA*, probably encodes a α-L-arabinofuranosidase, based on the strong similarity observed between the primary structure of its putative product and other bacterial arabinosidases. Whether this enzyme is extracellular or intracellular is unknown.

Expression of the *ara* operon is induced by L-arabinose and driven by a promoter located upstream of *araA*. This has been demonstrated in this study by Northern blotting and primer extension analysis. Examination of the *ara* operon promoter reveals −35 and −10 sequences, relative to its transcriptional start site (shown in Fig. 1), separated by an optimal spacing of 17 bp, identical to the consensus sequences derived from the analysis of many σ^A-dependent promoters (Moran *et al.*, 1982). These sequences were shown to be important for the interaction of σ^A with their cognate promoters (reviewed in Moran, 1993). The presence of a strong promoter raises the possibility that transcription of *ara* is negatively regulated like in other well characterized *B. subtilis* catabolic operons, such as *xyl* (Gärtner *et al.*, 1992) and *gnt* (Fujita & Fujita, 1987); in fact the product of *araC* recently cloned, is a negative regulator of the *ara* operon (I. Sá-Nogueira & L. J. Mota, unpublished). To characterize the regulation of *ara* expression in greater detail we constructed transcriptional fusions of the *ara* promoter to the *E. coli lacZ* gene in Ara^+ and Ara^- strains. The induction by L-arabinose in the Ara^+ background was approximately 100-fold and the pattern of expression observed in Ara^- and Ara^+ strains was very similar. Interestingly however, the levels of accumulated β-galactosidase activity in the Ara^- background were less than 60% of the fully induced level in the wild-type strain. Since in this strain the *ara* transcription unit is interrupted at the level of *araB* (Fig. 1), and a role in the transport of L-arabinose was proposed for the downstream genes *araN*, *araP* and *araQ*, this effect could be due to less accumulated intracellular L-arabinose which prevents full expression of the *ara* promoter. Another possible explanation is that the products of *araL* and *araM* could stimulate transcription from the *ara* promoter. Addition of glucose reduced the level of expression to less than 12% in both Ara^+ and Ara^- backgrounds, indicating that repression of the *ara* operon by glucose acts at the transcriptional level.

The regulatory system mediating catabolite repression in *B. subtilis* seems to be accomplished by a negative regulatory mechanism (reviewed in Hueck & Hillen, 1995; Saier *et al.*, 1996). This evidence is based on the location and the sequences of *cis*-acting sites (CREs) responsible for catabolite repression of several *B. subtilis* genes and operons. Moreover, catabolite repression of most genes regulated via these *cis*-acting sites is also affected by the *trans*-acting factors CcpA, a DNA-binding protein, and HPr, an intermediate in the phosphotransferase sugar transport system. It has been proposed that HPr-Ser-P might interact with CcpA and that this interaction might allow CcpA to bind to the CRE (Deutscher *et al.*, 1994). Strong evidence for this proposal, but also contradictory results, have been obtained recently (Saier *et al.*, 1996; and references therein). CREs of catabolic genes and operons are located either in the promoter regions, where the binding of a regulatory protein probably interferes with transcription initiation, or in the downstream regions (reviewed in Hueck & Hillen, 1995). In the case of the *hut* operon two active CREs were found, one at the promoter and the other within *hutP*, and a looping mechanism involving co-operatively bound CREs has been proposed to interfere with transcription initiation (Wray *et al.*, 1994). Furthermore, the transition-state regulator AbrB is capable of specifically binding to *hut* CRE *in vitro* and an *abrB* null mutation leads to more efficient catabolite repression of some genes in *B. subtilis*, including L-arabinose isomerase. Thus, AbrB has been suggested to compete for binding to CRE with CcpA (Fisher *et al.*, 1994). The promoter region of the *ara* operon contains a sequence very similar to the CRE consensus sequence (TGWNANCGNTNWCA; W = A, T; Weickert & Chambliss, 1990) located between the transcription start site and the translation start site of *araA* (position 191–204, Fig. 1). A second sequence, which shows weak similarity with the CRE consensus sequence was found within *araA* (position 260–273, Fig. 1). Since inducer exclusion does not play a major role in carbon regulation of expression of the *ara* metabolic genes (Sá-Nogueira *et al.*, 1988), as observed in the *hut* operon (Chasin & Magasanik, 1968), it will thus be interesting to investigate the role of CcpA, HPr and AbrB in the catabolite repression of the *ara* operon and whether these sequences are *cis*-acting sites responsible for catabolite repression of the *ara* genes.

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L-Ribulose 5-Phosphate 4-Epimerase from *Aerobacter aerogenes*

EVIDENCE FOR A ROLE OF DIVALENT METAL IONS IN THE EPIMERIZATION REACTION*

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SUMMARY

L-Ribulose 5-phosphate 4-epimerase from *Aerobacter aerogenes* was inactivated by treatment with EDTA and was reactivated to varying extents by the addition of divalent metal ions in the order: $Mn^{++} > Co^{++} > Ni^{++} > Ca^{++} > Zn^{++} > Mg^{++}$. When optimal Mn^{++} was present, the homogeneous enzyme had a specific activity of 70 μmoles^{-1} min^{-1} mg of protein at 28° and pH 7.2. This value is about five times greater than that displayed by the crystalline enzyme as isolated and assayed in the absence of added metal ion.

In other mechanistic studies, L-ribulose 5-phosphate 4-epimerase was found to be stable to treatment with sodium sulfite and arsenite in the presence of a thiol compound. It was also stable to sodium borohydride in the presence or absence of substrate. Further, a reaction of tetranitromethane with the enzyme-substrate complex could not be detected. Possible mechanisms for L-ribulose 5-phosphate 4-epimerase are discussed.

L-Ribulose 5-phosphate 4-epimerase from *Aerobacter aerogenes*, which catalyzes the interconversion of L-ribulose-5-P and D-xylulose-5-P, is unique among 4-epimerases in that it neither contains nor requires NAD^+ for catalysis (1). In addition, no evidence was found for the presence of chromophoric substances in the crystalline enzyme, nor did additional cofactors have an influence on the activity (1). In contrast, there is substantial evidence that epimerization by UDP-glucose 4-epimerase involves an oxidation-reduction mechanism utilizing NAD^+ as the electron acceptor and donor (2–6). Thus, if L-ribulose-5-P 4-epimerase catalyzes a similar oxidation-reduction reaction, another as yet unrecognized electron acceptor must perform the function of NAD^+.

It has also been observed that there is no kinetic isotope effect when D-[4-T]xylulose 5-phosphate is used as substrate (7). This is in contrast to UDP-glucose 4-epimerase where a normal isotope effect is observed (8). In this connection, it may be significant that the substrates, L-ribulose-5-P and D-xylulose-5-P, are open chain carbohydrates lacking a nucleotide moiety and possessing a carbonyl group two carbon atoms removed from the epimerization site. Undoubtedly, this confers chemical properties on the substrate which are considerably different from those of nucleotide sugars. For these reasons, mechanisms of 4-epimerization of L-ribulose-5-P other than oxidation-reduction have been considered.

The results presented in this paper indicate that L-ribulose-5-P 4-epimerase requires divalent metal ions for activity, and that different divalent metal ions activate to varying extents. In addition, an exploration of a number of mechanistic possibilities involving oxidation-reduction, or carbanion and carbonium ion formation, gave negative results.

MATERIALS

Chemicals—L-Ribulose-5-P was prepared according to the procedure of Anderson (9). Spectro-pure sulfate salts of Mn^{++}, Mg^{++}, Ni^{++}, Zn^{++}, and Co^{++} were obtained from Johnson, Mathley and Co., Ltd. Chloride salts of the divalent metal ions were obtained from Mallinckrodt, Inc. Tris base was obtained from Sigma Chemical Co.

Enzymes—L-Ribulose-5-P 4-epimerase from *A. aerogenes* (constitutive for L-arabinose operon, uracil auxotroph designated "u$^-$i$^-$") and D-xylulose-5-P phosphoketolase from *Leuconostoc mesenteroides* were purified by procedures previously reported (1). A triose phosphate isomerase-α-glycerol phosphate dehydrogenase mixture was obtained from Calbiochem.

METHODS

L-Ribulose-5-P 4-Epimerase Assay—The 4-epimerase was assayed by two methods designated as "continuous" and "two-step." The continuous assay involved the conversion of L-ribulose-5-P to α-glycerol phosphate with the concomitant oxidation of NADH utilizing phosphoketolase, triose phosphate isomerase, and α-glycerol phosphate dehydrogenase as coupling enzymes (1). The two-step assay involved the epimerization of L-ribulose-5-P to D-xylulose-5-P in Tris-Hepes[1] buffer, pH 8.0, in the absence of coupling enzymes. The 4-epimerase was then inactivated by the addition of acetic acid and heating in a boiling water bath for 1 min. The pH was readjusted to 7.0 by the addition of ammonium hydroxide, and an aliquot of the

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[1] The abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

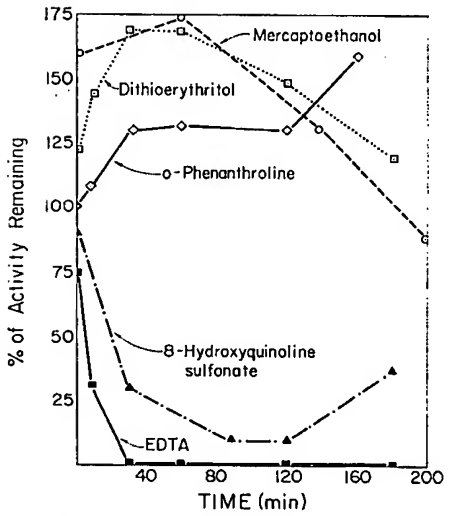

FIG. 1. The effect of metal chelators on enzyme activity. The 4-epimerase preparation, approximately 50% pure, was dialyzed against 0.05 M glycylglycine buffer, pH 8.0, and incubated at room temperature in 0.05 M glycylglycine, pH 8.0, with 1 M mercaptoethanol, 10^{-1} M dithioerythritol, 1 mM *o*-phenanthroline, 8×10^{-2} M 8-hydroxyquinoline sulfonate, or 10^{-3} M EDTA. An aliquot was withdrawn at the times indicated and assayed for 4-epimerase activity in the two-step assay at 37° and pH 8.0, as described under "Methods." Contaminating metal ions were not removed from reagents or glassware used in these assays. All values are expressed as percentage of the activity remaining compared to the activity prior to the addition of the chelator.

assay mixture was assayed for D-xylulose-5-P by measuring the amount of NADH oxidized upon enzymatic conversion of D-xylulose-5-P to α-glycerol phosphate in the presence of phosphoketolase, triose phosphate isomerase, and α-glycerol phosphate dehydrogenase (10). Only small amounts of metal or chelator from the incubation with epimerase was carried over in the analytical step for D-xylulose 5-phosphate. In separate controls it was shown that both the metal ions and chelators used affected the D-xylulose 5-phosphate values less than 10%.

Where indicated, all traces of metal ions were removed from the reagents and glassware used in the first step of the two-step assay. The glassware was soaked overnight in 4 N HCl and then was extensively rinsed with double quartz-distilled water. The buffers used in the assay were passed over Chelex resin and the pH was adjusted with solid Tris base. The L-ribulose-5-P was also passed through a Chelex column, and the pH was adjusted to 6.0 with Tris base prior to use.

One unit of enzyme activity is defined as the amount required to epimerize 1 μmole of L-ribulose-5-P per min at 28° and pH 7.2.

Protein concentration was determined using $A_{280}:A_{260}$ ratio method of Warburg and Christian (11).

Removal of EDTA by Chromatography on Sephadex G-25—EDTA was removed by passing the enzyme through a Sephadex G-25 (0.6 × 11 cm) column. The column, of sufficient length to clearly separate a mixture of blue dextran and ^{32}P, had previously been washed free of metal ions with 10^{-2} M EDTA followed by extensive washing with double quartz-distilled water. Prior to use, the column was equilibrated with Tris-Hepes buffer which had been freed of metal ions by passage through Chelex resin. All of the glassware used had been soaked overnight in 4 N HCl and extensively washed with double quartz-distilled water.

Tests for Lipoate and Cystine—L-Ribulose-5-P 4-epimerase (0.4 μmole, 85% pure) was incubated at room temperature with 10^{-1} M, 10^{-2} M, and 10^{-4} M Na_2SO_3 in 0.05 M Tris-Hepes buffer, pH 8.0. The activity remaining after 30 min was determined using the continuous assay as described under "Methods." Alternatively, the enzyme was incubated as above with 10^{-2} M mercaptoethanol or 10^{-3} M dithiothreitol. After 30 min, sodium arsenite was added to a concentration of 10^{-1} M to 10^{-4} M. Aliquots of the enzyme were removed after 5 min and the activity determined in the continuous assay.

RESULTS

Metal Ion Activation

Effect of Metal Chelators on L-Ribulose-5-P 4-Epimerase Activity—Although L-ribulose-5-P 4-epimerase does not require added organic or metal cofactors for activity, it is possible that tightly bound metal ions may participate in catalysis. As a first test of this hypothesis, the 4-epimerase was incubated with a series of metal chelators for various incubation times (Fig. 1). A wide variety of responses was observed including both inhibition and stimulation of activity. Complete inhibition was obtained only with 10^{-3} M EDTA. Although 2×10^{-3} M 8-hydroxyquinoline sulfonate did not alter the enzyme activity (not shown), 8×10^{-2} M 8-hydroxyquinoline sulfonate inhibited 90%. 2,3-Dimercapto-1-propanol (10^{-3} M) and mercaptoethanol (10^{-1} M) caused 30 to 60% loss in activity over the 2-hour period (also not shown in Fig. 1).

In contrast, an initial activation was obtained with either dithiothreitol, 1 mM *o*-phenanthroline, or 1 M mercaptoethanol. These results suggest that the enzyme, as isolated, may bind a variety of metals including those species which inhibit. Thus, EDTA may inactivate by removing all metal ions, whereas other metal chelators such as *o*-phenanthroline may activate by preferentially complexing certain inhibitory metal ions. This possibility is supported by the fact that the stability constants for Mn-*o*-phenanthroline or Mn-8-hydroxyquinoline chelates are two or more decades lower than those for heavy metals, whereas the Mn-EDTA stability constant is extremely high and in the same range as those for the heavy metals (12). However, the possibility that the metal chelators may nonspecifically activate or inactivate by means other than removal of a metal ion must be considered.

Activity of L-Ribulose-5-P 4-Epimerase after Removal of EDTA—In order to determine whether inactivation of the L-ribulose-5-P 4-epimerase by EDTA was due to chelation of metal ions or to binding of EDTA to the enzyme, it was necessary to determine the activity of the treated enzyme after removal of the EDTA. For this purpose, the enzyme was inactivated by incubation with 10^{-2} M EDTA for 1 hour at room temperature. The EDTA was then separated from the enzyme by passage through a Sephadex G-25 column as described under "Methods." All buffers used to elute the enzyme from the column and used in the enzyme assay were treated to remove trace contaminations of metal ions as described under "Methods."

The enzyme activity recovered from the Sephadex column varied from 0 to 10% of the initial activity. In the experiment cited in Table I no activity remained. In other cases where low activity remained, it was not ascertained how much was attributable to inaccuracies of the two-step assay, incomplete removal of metal, or recontamination by metal during passage

through Sephadex. At any rate when metal ions were added to the first step of the two-step assay, the activity was greatly increased; the increase depended upon the metal ion species present as detailed below.

Divalent Metal Ion Specificity—To determine the activating capability of various metal ions, the 4-epimerase was dialyzed overnight against 0.05 M Tris-Hepes buffer, pH 8.0, treated with EDTA, freed of EDTA on Sephadex G-25, and assayed in the presence of varying quantities of specific divalent metal ions as described under "Methods." The metal salts used were freshly prepared solutions of spectrographically analyzed metal salts containing less than 5 ppm of most other metals. Under the conditions and concentrations used no precipitation of Mn was observed either in reagents or incubation mixtures. Since, in a preliminary test, the same activity was obtained when the enzyme was previously incubated with 10^{-3} M Co^{++} for 0, 10, or 30 min, the enzyme was not previously incubated with metal ions prior to assaying. Rather, metal ions and substrate were incubated to allow temperature equilibration of the assay mixture, and the reaction was started by the addition of a very small volume of the 4-epimerase.

The results in Table I show that dialysis against Tris-Hepes buffer resulted in an activity loss of about 3-fold, presumably due to loss of metal ion. Following treatment with EDTA and passage through Sephadex G-25 no activity remained. After incubation with metals the highest 4-epimerase activity was obtained with Mn^{++}, and this activation occurred at the lowest divalent cation concentration. A 17-fold stimulation over the activity present in the dialyzed preparation was observed. The $MnSO_4$ concentration was almost optimal at 10^{-5} M (17-fold *versus* 18-fold stimulation at 10^{-3} M), whereas 10^{-4} M $NiSO_4$ and 10^{-3} M or higher $MgSO_4$ were required for the maximum activation. Similar activating effects were obtained using Cl^- salts of metal ions, thus indicating that a specific anion is not required.

To show the importance of EDTA treatment in obtaining full activation as described above, the 4-epimerase (90% pure) was dialyzed for 2 hours against 0.05 M barbital buffer, pH 8.0, then incubated for 1 hour with 10^{-2} M Co^{++}, Mn^{++}, Zn^{++}, and $MgCl_2$ salts, and assayed in the two-step assay. Contaminating metals were not removed from the glassware or reagents. The results presented in Table II indicate that only Mn^{++} can stimulate 4-epimerase which had not been treated with EDTA. However, only a 2-fold stimulation was obtained, indicating that Mn^{++} was not able to activate completely without prior EDTA treatment. These results suggest that various nonactivator divalent cations are bound to the Mn^{++} binding site of the 4-epimerase as isolated. These dissociate slowly even in the presence of Mn^{++}, as reported for phosphoglucomutase by Ray (13).

Specific Activity of Crystalline L-Ribulose-5-P 4-Epimerase in Presence of Mn^{++}—Since the preceding results strongly indicated that L-ribulose-5-P 4-epimerase was activated by metal ions, Mn^{++} being the most active, it was necessary to redetermine the specific activity of homogeneous Mn^{++} 4-epimerase.

The L-ribulose-5-P 4-epimerase was twice crystallized as previously reported (1). The second crystals were at least 98% pure as determined by polyacrylamide gel electrophoresis. The enzyme solution was then incubated with 10^{-2} M EDTA, and the EDTA was removed by passage through a Sephadex G-25 column as before. The metal-free enzyme was incubated with 10^{-4} M $MnSO_4$ (spectro-pure) and assayed with the two-step assay to which 10^{-5} M $MnSO_4$ had been added. A specific activity of 70 ± 7 units per mg of protein was obtained for the pure L-ribulose-5-P 4-epimerase, as compared with 12 units per mg of protein for the crystalline enzyme not so treated (1).

TABLE I

Divalent metal ion activation of L-ribulose-5-P 4-epimerase

The 4-epimerase (85% pure) was dialyzed overnight against 0.05 M Tris-Hepes buffer, pH 8.0, incubated for 1 hour with 10^{-2} M EDTA, and passed through a Sephadex G-25 column (0.6 × 11 cm) which had been washed free of cations with EDTA and equilibrated with 0.05 M Tris-Hepes buffer, pH 8.0. Activity was determined in the two-step assay containing spectro-pure metals at the levels indicated in the table. Precautions were taken to remove the contaminating metals from the glassware and the reagents as described under "Methods."

Conditions	Activity	Activation[a] at metal ion concentration of			
		10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
	units/mg protein				
Original enzyme	10.0				
After dialysis	3.4				
After EDTA treatment	0.25				
After Sephadex G-25	0.0				
$MnSO_4$		15	17	17	18
$CoSO_4$		9.4	11	15	18
$NiSO_4$		0.65	1.3	6.2	5.3
$CaCl_2$[b]			2.4		2.8
$ZnSO_4$		0.27	1.1	0.97	1.0
$MgSO_4$		0.11	0.09	0.09	0.38

[a] Expressed as -fold activation over the original activity.
[b] $CaCl_2$ was Mallinckrodt analytical reagent grade.

TABLE II

Effect of divalent metal ions on L-ribulose-5-P 4-epimerase

The L-ribulose-5-P 4-epimerase (specific activity, 10.0) was dialyzed for 2 hours against 0.05 M barbital buffer, pH 8.0, and incubated for 1 hour with 10^{-2} M Co^{++}, Mn^{++}, Zn^{++}, and Mg^{++} as chloride salts and assayed in the two-step assay in the presence of glycylglycine buffer, pH 8.0.

Additions	Original activity
	%
$CoCl_2$	89
$MnCl_2$	205
$ZnCl_2$	13
$MgCl_2$	78

Mechanistic Studies

Effect of Arsenite and Sulfite—Since the 4-epimerase is devoid of NAD^+, it was considered possible that the epimerization process may involve an oxidation-reduction mechanism using enzyme-bound oxidized lipoic acid or cystine as an electron acceptor. If this were true, either arsenite or sulfite should inhibit the 4-epimerase since dihydrolipoate and cysteine irreversibly react with sulfite.

Accordingly, L-ribulose-5-P 4-epimerase (85% pure) was incubated with 10^{-1} M, 10^{-2} M, 10^{-3} M, and 10^{-4} M sodium sulfite or sodium arsenite with and without prior incubation with either 10^{-2} M mercaptoethanol or 10^{-3} M dithiothreitol to reduce any

disulfide bonds, as described under "Methods." The epimerase was inactivated no more than 10% in any of these experiments, as determined in the continuous assay.

Effect of Sodium Borohydride Treatment—Less than 20% of the epimerase activity was lost on incubation with $NaBH_4$ in the presence or absence of substrate using the method of Ingram and Wood (14). In addition, when the 4-epimerase was incubated with borohydride in the presence of L-ribulose-5-P and 10^{-4} M $CoCl_2$, there was less than a 10% loss in activity. Likewise, the 4-epimerase at pH 6.5 in 0.05 M phosphate buffer was not inactivated by borohydride either in the presence or absence of substrate. The conditions used in these experiments are routinely used by others in this laboratory to obtain complete inactivation of 2-keto-3-deoxy-6-phosphogluconic aldolase in the presence of pyruvate, or of the pyridoxal phosphate-containing L-threonine dehydrase.

Test for Carbanion Intermediate—Carbanions react with tetranitromethane with the liberation of nitroformate which absorbs at 350 nm. Christen and Riordan (15, 16) have used this reagent to demonstrate the presence of a carbanion intermediate in both the yeast (Class II) and the muscle (Class I) fructose diphosphate aldolase-catalyzed reactions. Thus, it is reasonable to assume that if 4-epimerization of L-ribulose-5-P and D-xylulose-5-P were proceeding via a carbanion intermediate, it should be detected by tetranitromethane.

Nitroformate was produced in the presence of either L-ribulose 5-phosphate alone or the 4-epimerase (80% pure) alone in imidazole, glycylglycine, and Tris buffers; pure 4-epimerase did not react with tetranitromethane in Tris buffer. However, the rate of the reaction with L-ribulose-5-P plus the 4-epimerase was not significantly greater than the sum of the individual rates of reaction. In addition, increasing the amount of enzyme did not increase the rate of the nitroformate formation. In a control with FDP aldolase, it was possible to obtain a net increase in 350 nm absorbance with the rate of reaction being dependent upon the aldolase concentration.

DISCUSSION

If L-ribulose-5-P 4-epimerase functions by a mechanism similar to that of UDP-galactose 4-epimerase, there must be a group on the enzyme capable of oxidizing the hydroxyl group on carbon atom 4 of the substrate. However, previous results have indicated that NAD^+ is not present and is not required for enzyme activity (1). In addition, there were not chromophoric groups as are characteristic of many prosthetic groups and coenzymes. Since the 4-epimerase requires only the addition of metal ions for activity, the putative oxidation-reduction mechanism would have to involve only metal ions and the constituent amino acids.

Cystine and lipoic acid have reduction potentials comparable to that of NAD^+ and, thus, could participate in the epimerization reaction. Presumably, the disulfide form would be required. Since sulfhydryl groups are often readily oxidized by air, the oxidized form could predominate in the active site. Although lipoic acid absorbs at 330 nm, its extinction coefficient is too low to have been readily detected in previous spectral studies (1). However, the evidence discussed below tends to eliminate the SH-disulfide oxidation-reduction mechanism. First, borohydride should reduce the disulfide bond with loss in activity. Concerning the possibility that the disulfide may have been quickly reoxidized prior to or during the assay, it has been observed that activity was not lost on incubation with 1 M mercaptoethanol for 1 hour followed by assay in the presence of 0.05 M mercaptoethanol; that is, under conditions which are usually sufficient to reduce and maintain the integrity of disulfide groups. Although 50% of the activity was lost on incubation with mercaptoethanol for an additional hour, the activity was not recovered on passage through the Sephadex column, suggesting that the activity loss was due to some phenomenon other than reduction of a disulfide bond at the active site. Second, arsenite should have reacted with the reduced disulfide and caused inactivation, and third, sulfite should have reacted with the disulfide group to form the stable sulfur-sulfonated derivative.

No data have been obtained to indicate that an indolenine intermediate derived from tryptophan functions as the electron acceptor in the 4-epimerization as reported by Schellenberg for alcohol dehydrogenase (17).

Consequently, the previous results (1) and those presented herein are not consistent with the electron-acceptor being NAD^+, cobamide coenzyme, lipoate, cystine, or an oxidized indolenine derivative of tryptophan.

In the absence of any substantial evidence for participation of an oxidizing group on the enzyme, it is necessary to consider other mechanisms for epimerization such as: (*a*) a Sn_2 (Walden) inversion at C-4; (*b*) carbon-carbon bond cleavage and re-forma-

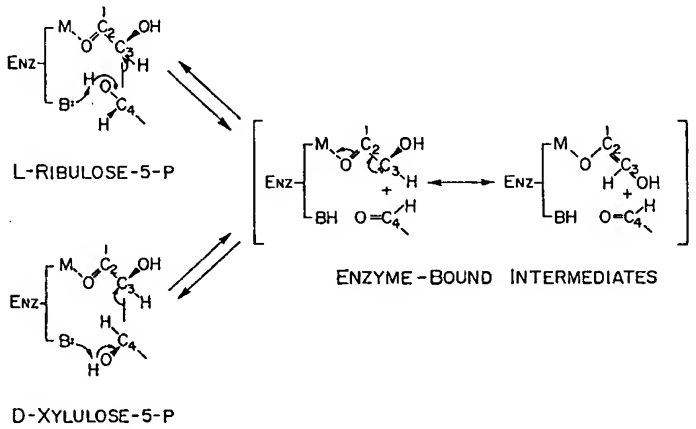

FIG. 2. Proposed dealdolization-aldolization mechanism for L-ribulose-5-P 4-epimerase. *M* depicts a divalent metal ion in the active site and *B*: indicates a base function in the active site.

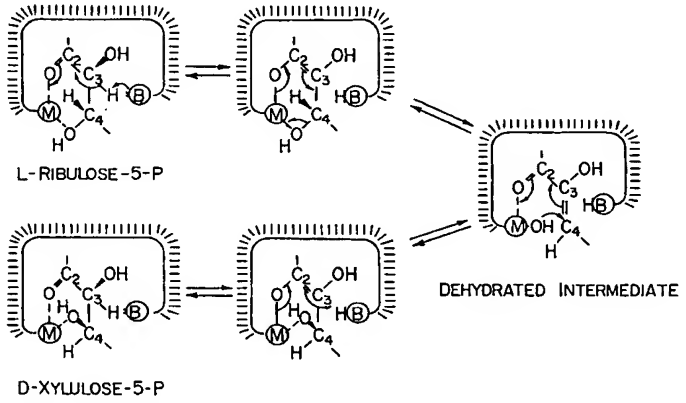

FIG. 3. Proposed dehydration-rehydration mechanism for L-ribulose-5-P 4-epimerase.

tion between C-3 and C-4; and (*c*) dehydration-rehydration at the same location. A Sn_2 inversion is not considered probable since McDonough and Wood (18) previously reported no isotope incorporation into L-ribulose-5-P and D-xylulose-5-P when the epimerization was conducted in $H_2{}^{18}O$.

The mechanism proposed in Fig. 2 depicts a metal ion-assisted aldolytic cleavage in a manner strictly analogous to the Schiff base mechanism (19, 20). The metal ion chelates with the carbonyl group (and possibly a hydroxyl group) and serves as an electrophile. A base on the enzyme surface acting as a nucleophile impinges on the C-4 hydroxyl group. In the ensuing rearrangement of electrons, C-3—C-4 cleavage occurs and C-3 takes on carbanion character. In completion of the rearrangement, a metal-oxygen bond is formed at C-2 along with a double bond at C-2—C-3. These intermediates would be analogous to the enamine and ketamine intermediates in the Schiff base mechanism. In this scheme, it is not intended to favor a discrete as opposed to a concerted mechanism.

If the characteristics of the epimerase are such that (*a*) the carbanion of dihydroxyacetone from carbon atoms 1, 2, and 3 cannot be discharged by a proton as in the case of transaldolase (21, 22) and (*b*) the glycolaldehyde phosphate moiety does not readily dissociate, it would follow that the carbon-carbon bond would immediately re-form. If there were a high probability that the bond between C-4 and the hydroxyl group would reform *cis* or *trans* in this process, epimerization would be observed. In such a mechanism, L-ribulose 5-phosphate 4-epimerase would, in fact, be a special kind of transaldolase to the extent that the dihydroxyacetone moiety does not dissociate. It would differ in that the other fragment, glycolaldehyde phosphate, is bound and precludes other aldehydes functioning in dihydroxyacetone transfer reactions.

If this hypothesis is correct, the carbanion intermediate would be very short lived because the proximity of glycolaldehyde phosphate would favor condensation. In this connection, the reaction of tetranitromethane in fructose diphosphate aldolase- and transaldolase-catalyzed reactions may be observable because dissociation of glyceraldehyde 3-phosphate allows access to the carbanion intermediates.

An alternative mechanism would be dehydration-redehydration by acid-base catalysis as shown in Fig. 3. The first step would involve a base-catalyzed removal of the proton on C-3 leaving either a carbanion at C-3 or a double bond between C-2 and C-3. The presence of the metal ion in the active site would facilitate removal of the C-4 hydroxyl group in a manner proposed for enolase (23) and aconitase (24). In the reverse reaction, the return of the hydroxyl group would have high probabilities of occupying either bonding position.

Since McDonough and Wood (18) were unable to find an incorporation of T or ^{18}O from the medium into the substrate, the limitation on this mechanism is that the same proton and hydroxyl groups removed must be involved in the reverse reaction. In this connection, Rose (25) has produced evidence with phosphoglucoisomerase that the intramolecular transfer of a proton can be faster than equilibration with the surrounding medium. Thus, it is conceivable that the proton removed from C-3 becomes bound to the enzyme and is not free to diffuse into the medium. The hydroxyl group would probably be chelated by the metal ion in a position where it would be readily accessible to both sides of C-4 but not to the medium.

Neither dealdolization-aldolization nor dehydration-redehydration can participate in the mechanism of the other carbohydrate 4-epimerases since the substrates of all other 4-epimerases do not possess a free carbonyl group which could participate in the mechanism.

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